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Platelets in Thrombotic and Non-Thrombotic Disorders

Pathophysiology, Pharmacology and Therapeutics: an Update

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Foreword

When I joined Marc Verstraete's laboratory as a medical student in 1959, the emphasis was on hemophilia, vitamin K antagonists as oral anticoagulants, and the development of thrombolysis. Platelet studies were limited to platelet count, bleeding time, and clot retraction. When an otherwise healthy patient with a bleeding disorder had a normal coagulation profile, a normal platelet count, a prolonged bleeding time, and an absent clot retraction, we diagnosed Glanzmann's thrombasthenia; if, however, in the same setting the clot retraction was normal, we considered von Willebrand's disease. How times have changed! In the 1960s, aggregometry allowed the first functional platelet studies; the inhibitory effect of some anti-inflammatory agents, in particular aspirin, was discovered, and the first speculations made about a possible antithrombotic potential of these agents. In the 1970s, there was in scientific meetings a strict divide between "clotters" (studying coagulation) and "clumpers" (studying platelet aggregation); fortunately, this gap gradually narrowed when it became obvious that platelets are closely involved in physiological coagulation and that coagulation induces platelet activation. The 1980s saw confirmation of aspirin as antithrombotic agent; it became a mainstay for the prevention and treatment of arterial vascular disease. At the same time, new developments in cell biology were being applied to megakaryocytes and platelets. Since then, new pathways and receptors have been continuously discovered; the molecular bases of numerous congenital thrombocytopenias have been defined; new platelet inhibitors have been developed and evaluated in clinical studies; platelet inhibition remains crucial despite the continuing progress with vascular stents to combat arterial disease. Platelets are now recognized to play an important role in inflammation, angiogenesis, cancer, etc.

So why, in this Internet age, a new book on platelets? Platelet studies are a typical area where cell biology and clinical practice meet. A book is an ideal site to bring these two separate worlds together. Cell biologists are interested in the potential clinical application of their findings; clinicians are interested in the biological basis of their treatments; this book provides all the answers in one place. It now has become impossible to possess the total knowledge on platelets; even experts will benefit from a book like this. For students entering the field, it provides them with a daunting but comprehensive "state of the art," to which they can hope to add new findings.

The first edition of this book dates from 2002. When comparing the Tables of Contents, I find, besides an update of previously covered topics, many new entries such as the platelet proteome and transcriptome, CLEC, a new emphasis on neutrophil–platelet interactions, platelets as carriers of genetic material for cell delivery, or platelets in regenerative medicine, among several others.

The book now contains 97 chapters. To identify and convince top scientists to contribute, to put this all together, to elaborate a useful index for search purposes, all this is

a formidable task. I wish to congratulate Paolo Gresele, with whom I shared an exciting research period and a continuing friendship, José Lopez, Neil Kleiman, and Clive Page for this hard work, but, above all, for a very useful achievement, which will bear many fruits in the years to come.

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Contents

Contents for Volume 2

Part I

Physiology

A Brief History of Blood Platelets: A Personal View

Michael C. Berndt, Pat Metharom, and Robert K. Andrews

Abstract

A search on 'platelets' in Pubmed yields more than 100,000 publications indicating both the depth and interest. Platelets are small anucleate cells that circulate freely in the vasculature. In haemostasis, they adhere rapidly, become activated, spread and coat the damaged vasculature. Our understanding of platelet structure and function has advanced in waves, dependent upon the development of the next cutting-edge technology. This brief chapter describes the key historical events leading to our current understanding of platelet structure and function.

Introduction

Platelets are small anucleate cells that circulate freely in the vasculature. In haemostasis, they adhere rapidly, become activated, spread and coat the damaged vasculature. The activated platelets become cohesive and release their dense body and alpha granule contents aiding in the recruitment of additional platelets and the formation of a platelet aggregate or thrombus that is stabilised by activation of coagulation and thrombin-dependent fibrin deposition (Andrews and Berndt 2004). Although haemostasis and thrombosis are now recognised not to be identical processes, a similar sequence of events also occurs in arterial thrombosis. Besides their pivotal functional role in haemostasis and thrombosis, platelets are now known to be essential in a wide variety of non-haemostatic functions, including their interface with inflammatory responses; in rheumatoid arthritis, cancer metastasis, tumour angiogenesis and lymphatic development; in the regulation of infection; and the killing of intra-erythrocytic malarial parasites, amongst others (Boilard et al. 2010; Erpenbeck and Schön 2010; Klement et al. 2009; Suzuki-Inoue et al. 2010; Clark et al. 2007; McMorran et al. 2009).

A definitive treatise on the history of platelet research, both from a basic and a clinical perspective, could easily be the subject of a multivolume series, rather than the subject of a brief chapter. At the time of writing, a search on 'platelets' in Pubmed yields more than 100,000 publications indicating both the depth and interest in the field. Those who are interested in a more structured and recent review on the history of platelet research, both from a basic and clinical viewpoint, and also from the perspective of where the field is leading, are referred to the excellent recent review by Coller (2011). I (MCB) started my career in platelet research in the laboratory of David Phillips in Memphis Tennessee in 1979. Like many fields of research, our understanding of platelet structure and function has advanced in waves, dependent upon the development of the next cutting edge technology. In David Phillips's laboratory, the interest was on the structure and function of platelet membrane glycoproteins. The

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then breakthrough platform technology was the capacity to surface label human platelet membrane glycoproteins and analyse them on one- and two-dimensional SDS-polyacrylamide gels. It is this technology that led to the definition of the membrane glycoprotein defects in Bernard-Soulier syndrome (BSS) and Glanzmann's thrombasthenia (GT) and the fundamental understanding of the role of these glycoproteins in haemostasis and thrombosis.

The Discovery of Blood Platelets and Early Research

Spanning centuries, the discovery of blood platelets was fundamentally dependent on the invention of the compound microscope by Hans and Zacharias Janssen in the 1590s (see Table 1). Discovery of blood platelets as a distinct cellular entity in blood is attributed to Bizzozero in 1882, although their discovery and potential function are prefaced in earlier studies over the previous 40 years (Table 1). In particular, the studies of George Gulliver and William Addison in the 1840s predated the first identification of blood platelets and their function with respect to their association with the fibrin clot (Fig. 1) (Addison 1842; Gerber and Gulliver 1842). In 1906, James Wright described the bone marrow and megakaryocyte origin of platelets (Wright 1906). By the late 1950s, the ultrastructure of platelets along with their constituent intracellular organelles such as alpha granules and dense bodies had been described as had the fundamental role of blood platelets in bleeding and thrombosis and their pivotal and necessary contribution to coagulation (Hellem and Owren 1964; Berger 1970; Coller 2011). The field awaited the transition from the observational to the molecular age of research.

Table 1 Evolution of knowledge leading to platelet discovery and its origin

Timeline	Discoveries	References
1590s	Hans and Zacharias Jansen invent compound microscope	Spencer (1937)
1628	Publication of *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* (*Movement of the Heart and the Blood in Animals*) by William Harvey demonstrates the concept of blood circulation	Kilgour (1961), Harvey and Leake (1928)
1665	The best-seller *Micrographia* from Robert Hooke details his discovery of cells	Hooke (1665)
1674	Anton van Leeuwenhoek (or Leeuwenhoeck), using his handcrafted and most advanced microscope of the period, gives the first accurate description of red blood cells	Leeuwenhoeck (1674)
1771	Publication of William Hewson's *Experimental Inquiry into the Properties of the Blood.* Discovery of fibrinogen and anticoagulants (neutral salts)	Hewson (1771)
1827	Joseph Jackson Lister, a pioneer achromatic objective lenses for the compound microscope, publishes *Notice of some Microscopic Observations of the Blood and Animal Tissues* with Thomas Hodgkin. It is now possible to resolve objects accurately to '1/3000' in. (approx. 8 μm) and without spherical aberration	Hodgkin and Lister (1827)
1840s	George Gulliver publishes one of the first illustrations of platelets. He describes minute spherules sized at 1/10,000 of an inch (approx. 2.5 μm) that can become star-shaped, although no significance is attached to this observation	Gerber and Gulliver (1842)
	William Addison describes within blood minute granules 8–10 times smaller than 'colourless corpuscles' in relation to fibrin clot	Addison (1842)
	Alfred Donné defines three elements within blood, red and white cells and small globulins, less than 1/300 mm (approx. 3 μm) in size, that look like globulins of chyle	Donné (1842)
1864	Lionel S. Beale publishes the first clear illustration of platelets in *On the Germinal Matter of the Blood, with Remarks upon the Formation of Fibrin*, although these colourless corpuscles were considered by Beale to be early stage of blood cell development	Beale (1864)
1865	Max Schultze defines a group of colourless little spherules 6–8 times smaller than RBCs as a distinct and normal blood constituent population	Schultze (1865), Brewer (2006)
1874	William Osler describes pale, round discs in blood that can quickly coalesce in shed blood. Unfortunately, Osler thought them to be a type of bacteria	Stone (2003), Osler (1873)
1882	Giulio (or Julius) Bizzozero coins the terms 'piastrine', 'plaquettes' and 'Blutplattchen' (Italian, French and German, respectively) to describe platelets and demonstrates the importance of platelets in thrombosis and as the third component of the blood (together with RBCs and WBCs)	Brewer (2006), Bizzozero (1881), Wright (1906)
1906	James Homer Wright demonstrates the bone marrow and megakaryocyte origin of platelets	Wright (1906)

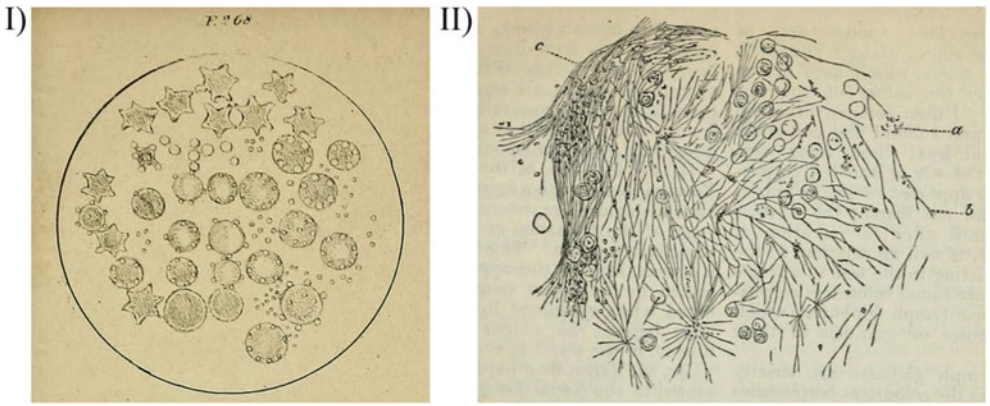

Fig. 1 (**a**) John Siddall's drawing of George Gulliver's minute spherules (Gerber and Gulliver 1842). (**b**) The illustration of William Addison's fibrin clot shows a delicate mesh of fibrin, blood cells and minute molecules or granules (*a*), filaments with molecules (*b*) and thick coagulated fibrin (*c*) (Addison 1842)

The 1960s

The 1960s was the decade in which most of our modern understanding of platelet function was initiated. This was launched on the basis of what was then a remarkable insight and innovation, the invention of the light transmission platelet aggregometer by Born in 1962 (Born 1962). This facilitated at this time or shortly afterwards the identification of what are now recognised as the primary physiologically relevant platelet agonists, ADP, collagen and thrombin (Born 1962; O'Brien 1962; Prentice et al. 1966; Zucker et al. 1966). By the end of the 1960s, the concept and mechanism for development of the platelet haemostatic plug were clearly identifiable in terms of modern concepts (Fig. 2) (Berger 1970).

The 1970s

The 1970s saw the discovery of the role of the regulatory arachidonic acid metabolites, thromboxane A2 and prostacyclin, as positive and negative regulators of platelet function, followed in the 1980s by the identification of nitric oxide as a negative regulator of platelet function (Hamberg et al. 1975; Moncada et al. 1976; Mellion et al. 1981; Palmer et al. 1987). The next historical advance in the understanding of platelet biology was the analysis of platelet–vessel wall interactions utilising new model systems developed by Baumgartner and colleagues as well as other investigators. Importantly, these studies took into account the effects of blood flows on platelet behaviour, with long-lasting consequences. When blood was flowed over rabbit aortas denuded of the endothelial lining by a balloon catheter, analysis by light or electron microscopy revealed a 'tight and continuous' layer of platelets within several minutes (Baumgartner and Haudenschild 1972; Baumgartner 1973). Further, this adhesion appeared to be independent of fibrin formation. These observations were in contrast to what was seen at lower blood flows in the same system where fibrin deposition was apparent at the subendothelial surface together with platelets and red blood cells. Together, this work pointed to a critical role for platelets in thrombus formation at elevated shear rates and independent of coagulation. However, specific platelet receptors and adhesive ligands relevant to high shear-dependent platelet adhesion were yet to be identified.

This detailed analysis of time-dependent morphometric changes of platelets interacting with subendothelium also showed a series of steps, from circulating platelets, to initial contact, adhesion and finally 'thrombus' formation (Baumgartner and Haudenschild 1972). The major platelet shape change associated with these stages was also notable. Further studies began to provide a foundation for subsequent identification of key platelet-specific receptors mediating these high-flow events. A highly negatively charged carbohydrate-rich layer (glycocalyx) on the surface of platelets containing factors critical to adhesion and aggregation was identified by electron microscopy and biochemical analysis of isolated platelet membrane fractions, notable both for the unique glycoprotein component and high-density sialic acid (Nurden 1974). Platelet aggregation induced by 'bovine FVIII' [now identified as bovine von Willebrand factor (VWF)] or by ristocetin and platelet adhesion to the subendothelial matrix were known to be defective in the rare congenital macrothrombocytopenia, Bernard-Soulier syndrome (BSS), whereas the response to other agonists was essentially normal. A 'gross abnormality in the 155,000-molecular weight glycoprotein seen in membrane fractions' of BSS platelets was identified as a key factor in the functional defect. This was supported using an acquired antiplatelet antibody isolated from a multiply transfused BSS patient (Nurden and Caen 1975; Tobelem et al. 1976). Treatment of normal platelets with this antibody in vitro resulted in a specific BSS-like aggregation defect. Subsequent detailed analyses by several groups identified the 155,000-molecular weight glycoprotein as GPIbα along with the absence of the other subunits of the GPIb-IX-V

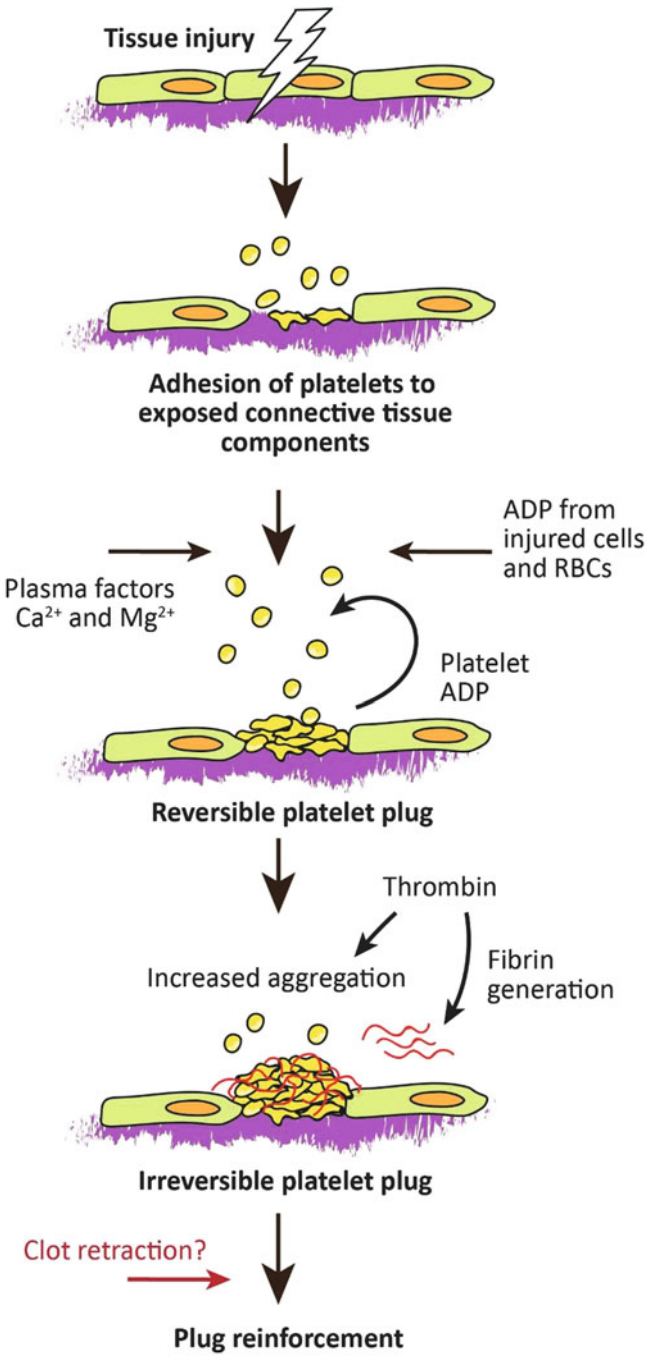

Fig. 2 The concept of the haemostatic plug circa 1960s (Hellem and Owren 1964; Berger 1970)

complex (Nurden et al. 1981; Clemetson et al. 1982; Berndt et al. 1983). These and other contemporary studies showing decreased adhesion of giant BSS platelets to subendothelium under flow conditions also further implicated a key role for VWF in platelet adhesion at elevated shear rates (Weiss et al. 1974, 1978). Importantly, an abnormal platelet glycoprotein pattern was also recognised in another inherited platelet disorder, Glanzmann's thrombasthenia (GT), where, unlike BSS, platelet aggregation was impaired to all agonists except ristocetin (Zucker et al. 1966; Nurden and Caen 1974). The analysis of platelets from healthy donors or patients with GT where membrane surface glycoproteins were labelled and separated by two-dimensional SDS-polyacrylamide gel electrophoresis revealed individual molecules and defects associated with two major glycoproteins in GT platelets ('IIb and III') (Phillips and Agin 1977). At this stage, specific platelet glycoprotein (GP) receptors were identified as functional, but biochemically were little more than spots on gels, with protein properties and regulatory mechanisms yet to be fully defined.

The 1980s and 1990s

The important role of specific platelet membrane glycoproteins as receptors for collagen, VWF and other adhesive ligands in platelet adhesion to subendothelium and in platelet function in general was further elucidated using improved in vitro perfusion systems (Baumgartner and Sakariassen 1985; Sakariassen et al. 1987; Fressinaud et al. 1988), by quantitative analysis of receptor–ligand interaction, by analysis of platelets deficient in particular receptors, by the generation of novel function blocking anti-GP antibodies and by specific inhibitors primarily isolated from snake venoms. For GPIIb-IIIa ($\alpha_{IIb}\beta_3$), the combination of these types of approaches identified fibrinogen, fibronectin and VWF as physiologically relevant ligands in platelet aggregation and the important role of the peptide sequence, Arg-Gly-Asp (RGD), in ligand recognition (Coller 1980; Peerschke et al. 1980; McEver et al. 1983; Coller 2015). For GPIb, analogous approaches defined the role of the N-terminus of the GPIbα chain of the GPIb-IX-V complex in binding von Willebrand factor and thrombin (Ruan et al. 1981; Coller et al. 1983; Fujimura et al. 1986; Andrews et al. 1989b; De Marco et al. 1994). A large family of RGD-containing venom proteins, termed disintegrins, were shown to act as potent inhibitors of platelet aggregation (Niewiarowski et al. 1994).

Indeed, snake toxins selectively targeting platelet glycoproteins and their ligands—both activating and inhibitory—played a central role in defining the function of platelet receptors. The ability of the C-type-like snake venom lectin, botrocetin, to induce VWF-dependent platelet aggregation had been investigated as a clinical laboratory test for assessing GPIb–VWF interaction since the early 1980s (Brinkhous et al. 1983; Howard et al. 1984). Botrocetin was subsequently purified (Andrews et al. 1989a; Fujimura et al. 1991) and contributed to new structure-function analysis of binding domains for VWF and GPIbα interaction (Sugimoto et al. 1991; Berndt et al. 1992). Isolation and identification of the primary platelet collagen receptor,

GPVI, was greatly facilitated by the snake venom toxin, convulxin, which induced potent collagen-like signalling/activation and bound p62/GPVI (Polgár et al. 1997). Toxins containing Lys-Gly-Asp (KGD) mimicking the RGD sequence in $\alpha_{IIb}\beta_3$ ligands were also intensively investigated as antithrombotic agents (Scarborough et al. 1991, 1993a, b, 1999), ultimately leading to the clinically used antiplatelet agent, eptifibatide (Integrilin), a cyclic heptapeptide. Other important snake venom toxins reported in the mid-1990s included mocarhagin, and subsequently Nk proteinase, metalloproteinases with exquisite selectivity for cleaving the sulphated anionic sequence of GPIbα, resulting in loss of binding of both VWF and thrombin. Mocarhagin also cleaved an analogous sequence of P-selectin glycoprotein ligand-1 (PSGL-1) and played an important role in mapping sites and defining specific functions of these receptors (De Luca et al. 1995; Ward et al. 1996; Wijeyewickrema et al. 2007; Ravanat et al. 2010; Carrim et al. 2015).

The functional analysis of platelet membrane glycoproteins was also greatly facilitated by the cloning of the individual receptors including GPIIb and GPIIIa, the GPIb α and β subunits, and GPVI (Poncz et al. 1987; Loftus et al. 1987; Fitzgerald et al. 1987; Lopez et al. 1987, 1988; Rosa et al. 1988; Clemetson et al. 1999), which, when expressed in cell lines, provided information on the regulation of surface expression and co-complexing with other receptors and signalling proteins. This included identification of additional binding partners beyond adhesive ligands involved in haemostasis and thrombosis, including GPIbα binding P-selectin and leukocyte $\alpha_M\beta_2$ (Romo et al. 1999; Simon et al. 2000). These approaches, together with the increased analysis of genetic models in mice, have helped characterise the pathophysiological roles of platelets not only in thrombosis and haemostasis but also in inflammation and other vascular processes (López et al. 2005) to where the field lies today.

Take-Home Messages

- A history of basic and clinical platelet research could be the subject of a multivolume series (>100,000 publications on 'platelets' in Pubmed).
- This brief chapter provides an individual investigator's perspective of key historical discoveries and developments in the platelet field.
- Advances in understanding have depended upon the ongoing development of new technologies.
- Over decades, the role of key platelet-specific glycoproteins in haemostasis and thrombosis has been defined, related to molecular defects in inherited platelet disorders.

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Phylogeny of Blood Platelets

Stefania Momi and Viroj Wiwanitkit

Abstract

In biology, phylogenetics is the study of the evolutionary history and relationships among individuals or groups of organisms (e.g., species or populations). At present, molecular phylogenetics is the modern technique for helping with the classification of biological systems. This chapter briefly discusses the phylogeny of blood platelets.

Introduction

In biology, phylogeny indicates the development or evolution of a particular group of organisms and can help to classify biological materials based on evolutionary relationships (Rosen 1974). Phylogenic classifications are based on anatomical appearance, physiology, biochemistry, as well as genetics. With the great advancements in genetic knowledge, modern phylogeny is largely based on the similarity or differences of genetic traits, i.e., on DNA or RNA. At present, scientists rely mainly on genetic phylogeny for classifying living organisms, and indeed using genetic information for classification is better than using anatomical, physiological, or biochemical features since the phenotype may be similar despite there being a different genotype. The use of genetics to study phylogeny is called phylogenetics (Marks 1991; Thornton 2002) and is based on the study of the genome to find out similarities between organisms, similar to dichotomous keys in classical taxonomy, thus defining the phylogenetic tree. The living organisms that have close relationships in evolution will be placed at a near distance in the phylogenetic tree (Fig. 1).

In addition to the classification of living organisms, phylogenetics is applicable to the classification of the components of organisms, including proteins, cell types, or biological structures, and of molecules, through the use of molecular phylogenetics (Marks 1991; Pyron 2015; O'Brien 1994).

An important advance in molecular phylogenetics has come from the introduction of bioinformatics. In fact, after the completion of the human genome project, computational and engineering approaches for the manipulation of data derived from genetic analyses have become available (Watson 1990; Cantor 1990) allowing in silico approaches to the study of structural and comparative genomics, the latter also applicable to phylogenetic studies (Eisen 1998; Eisen and Wu 2002). Phylogenomics involves reconstruction analysis of gene function and evolutionary analysis of rates and patterns of gene evolution (Eisen and Wu 2002).

Phylogeny of Platelets

Although once primarily known for their role in hemostasis and thrombosis, platelets have been increasingly recognized as multipurpose cells. Indeed, they have the ability to influence a wide range of seemingly unrelated pathophysiological events, like hemostasis, lymphogenesis, tissue regeneration, infection, and immunity (Franco et al. 2015) (see current book).

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The ability of platelets to exert a wide repertoire of complex functions likely dates back to an era in which innate host defenses were accomplished by one cell type, or at most by a small number of cells, instead than by the extensive and specialized cellular repertoire that has then developed in the blood of humans and other mammalian species (Fig. 2).

Invertebrate species generally possess an open circulatory system (the hemolymph) and lack differentiated formed elements in blood, such as neutrophils, erythrocytes, and platelets. Indeed, in many marine invertebrates (ascidians, mussels, decapod crustaceans, marine arthropods, etc.), only one type of cell circulates in blood or is present in the coelomic fluid, the fluid contained in the main body cavity in most multicellular invertebrates, and plays multiple roles in host defense, among which hemostasis. Such cells can aggregate and seal wounds (Levin 2002). One example is the amoebocyte of the arthropod horseshoe crab, *Limulus polyphemus*, that inhabits coastal waters of North America. In horseshoe crabs, one of the major defense systems is carried by the hemolymph which contains at least two types of hemocytes, granular and non-granular, based on cell morphology. However, there appears to be only one type of hemocyte in the systemic circulation of the adult animal, the so-called amoebocyte/granulocyte characterized by an oval, plate-shaped structure, 15–20 μm in its longest dimension that contains numerous dense granules since the population of the non-granular hemocytes is only 1 % of the total cells (Iwanaga and Kawabata 1998). These cells have primitive wound-sealing functions, aggregate in response to lipopolysaccharide (LPS), and release a cascade of antimicrobial substances (such as the anti-LPS factor), lectins, protease inhibitors (including trypsin inhibitor and α_2-macroglobulin), and also coagulation factors (factor C, factor B, factor G, proclotting enzymes, coagulogen) involved in the engulfing and killing of invading microbes and in preventing the leakage of hemolymph (Smith et al. 1995; Levin and Bang 1964; Takahashi et al. 1994; Goffinet and Grégoire 1975). The granular hemocytes of the horseshoe crab *Tachypleus tridentatus* have similar activities, although divergence of the two species appears to have occurred over 100 million years ago, suggesting that the granular hemocyte has

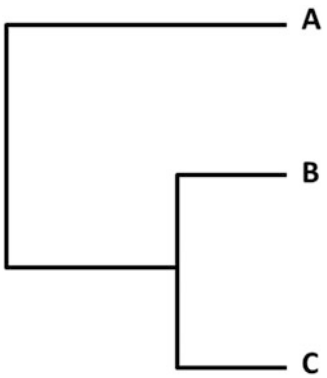

Fig. 1 Example of a phylogenetic tree. From this tree, C is closer in genetic evolution to B than A

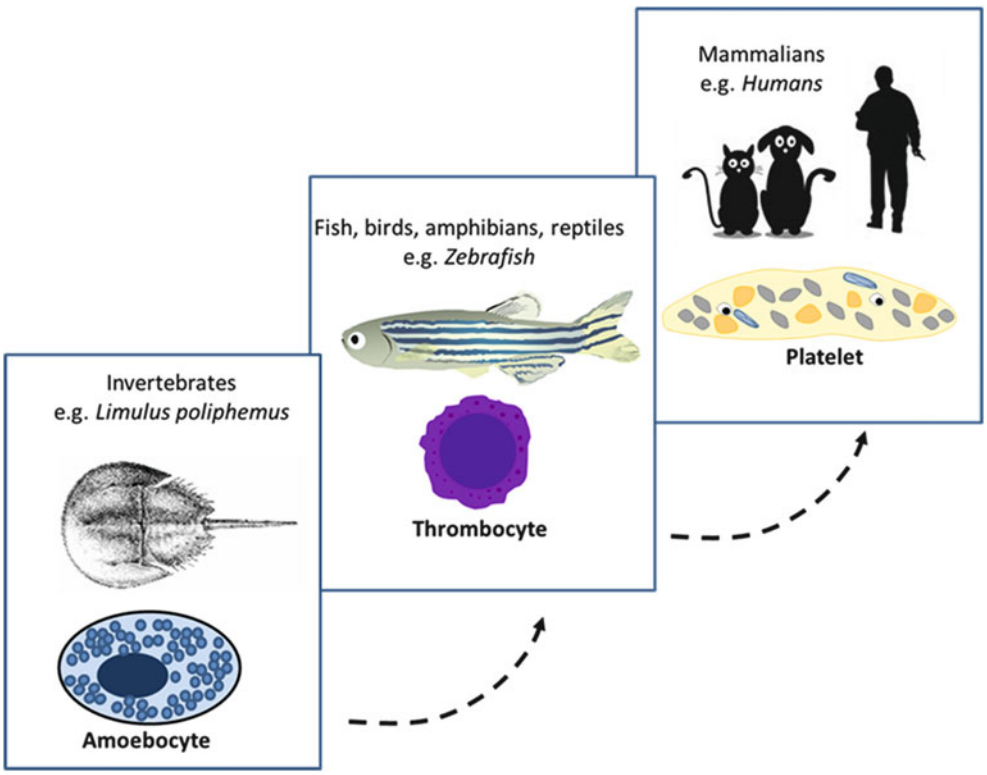

Fig. 2 Evolutionary links of platelets to multifunctional innate defensive cells provided with primitive multiple defensive roles

developed earlier than the divergence between the two species (Kawabata et al. 2003). Drosophila largely relies on innate immunity, encompassing both a humoral response of antimicrobial peptide expression and cellular responses for bacteria encapsulation and phagocytosis (Royet et al. 2003). At every developmental stage of *Drosophila*, aside from the early embryo, more than 90 % of all hemocytes are plasmatocytes (Rizki and Rizki 1984), which have important functions during animal development and in response to infection, tissue damage, and tumor growth. The mechanisms that coelomocytes employ to clear microbes from the coelom require shape change and therefore a cytoskeleton and include activities as motility, phagocytosis, secretion, degranulation, and clot formation (Smith et al. 1995). Sea urchin coelomocytes accumulate at sites of injury in response to chemotactic stimuli, generate sealing and encapsulating clots that are dependent on cell–cell adhesion, and have secretory and phagocytic functions (Rothenberg and Davidson 2003). Profilin is an important regulator of actin filament polymerization in sea urchins and becomes activated through the inositol triphosphate second messenger system.

Coelomocytes of earthworms have many similar functions (Cooper et al. 2002). These and other examples of invertebrate cellular responses provide key insights into the evolution of vertebrate innate and acquired host defenses (Salzet 2001).

Nonmammalian vertebrates have nucleated, often spindle-shaped, thrombocytes that represent the first cells that specialized in hemostasis during evolution (Ratnoff 1987). Zebrafish (*Danio rerio*) and other fish species have circulating mononuclear leukocytes, granulocytic leukocytes, and nucleated thrombocytes that have phenotypic features and functional characteristics similar to those of platelets (Rowley et al. 1997; Jagadeeswaran et al. 1999). However, in contrast to platelets, thrombocytes do not aggregate in response to adenosine diphosphate (ADP), nor to epinephrine (Belamarich et al. 1966; Ratnoff 1987), and, in contrast to mammalian platelets, they do not accumulate or produce serotonin.

However, Zebrafish thrombocytes play a role in the development of arterial thrombi (Thattaliyath et al. 2005). Thrombocyte aggregation in sharks is temperature reversible, a feature that is not seen with mammalian platelet aggregation (Rowley 1988). Shark thrombocyte aggregation is also independent of thrombin and ADP (Rowley 1988). Thrombocyte concentration and clotting time appear to be negatively affected by glucocorticoid excess in fish. Excessive levels of glucocorticoid tend to decrease the total thrombocyte count and increase the clotting time. Reptiles, birds, and amphibians also have nucleated thrombocytes (Levin 2002). Thrombocyte cytoplasmic granules have been described as numerous, scarce, or absent, depending on the vertebrate group or species (Lopez-Ruiz et al. 1992; Esteban et al. 2000). For example, only a single population of granules has been reported in fish granular thrombocytes and in amphibian thrombocytes. These granules show morphological and cytochemical features typical of lysosomes, although the presence of a variable number of cytoplasmic vesicles has led to the suggestion that subpopulations exist in fish (Pica et al. 1983). Occasional azurophilic granules have been described in reptile thrombocytes (Sypek and Borysenko 1988). Moreover, in the thrombocyte cytoplasm and lower vertebrate blood plasma, the presence of factors similar to those present in platelets has been shown (e.g., platelet factor 4, β-thromboglobulin, factor VIII-related antigen, etc.) (Spurling 1981; Pica 1990; Taffarel and Oliveira 1993; Hill and Rowley 1998). The cytoplasm in reptile thrombocytes is particularly delicate, viscous, and prone to rupture (Sypek and Borysenko 1988). Their perinuclear area contains actin-like filaments, and a smooth endoplasmic reticulum is sparsely distributed throughout the peripheral cytoplasm (Wood and Ebanks 1984). Concerning birds, an extensive ultrastructural study of six domestic species showed that thrombocytes are similar in size to lymphocytes, but have a denser nucleus and a very highly vacuolated cytoplasm. Moreover, they have a membrane surface-connected canalicular system similar to mammalian platelets (Levin 2002). Thrombocytes are the most common blood cell in the chicken after erythrocytes; they contain 5-hydroxytryptamine (Kuruma et al. 1970) and release protein materials containing a well-separated fraction which seems to correspond to β-thromboglobulin (Wachowicz and Krajewski 1979). Birds have more prolonged bleeding and do not form vaso-occlusive thrombi after arterial vessel wall injury in vivo (Schmaier et al. 2011). A recent study comparing a set of 5 representative mammals with 48 bird species across the avian phylogeny, 12 reptiles, 3 amphibian, and 3 fish species showed that the cytoplasmic domain of GPIbα is lacking in birds and this might help to explain why, unlike mammalian platelets, avian thrombocytes take longer to produce a clot to control bleeding (Ribeiro et al. 2015).

Some functions of mammalian platelets are vestiges of activities originally present in the primitive multicompetent cells from which mammalian blood cells have evolved. Nonnucleated platelets, and presumably their polyploid megakaryocyte progenitors in the bone marrow, are present only in mammals, suggesting that this unique mechanism producing an unprecedented, highly specialized anucleated cell from the cytoplasm of a larger cell allows to provide more evolved organisms with a highly efficient system supporting hemostasis. However, although the platelet is highly differentiated for *hemostatic* defensive functions, it maintains potent *inflammatory* activities and molecular mechanisms that link the hemostatic and immunologic/inflammatory systems, possibly as a rudimentary remnant from the more primitive multifunctional innate defensive cells. The evolutionary events that resulted in the appearance of mammalian megakaryocytes and platelets, as well as the potential biological advantage of this system, remain to be further clarified.

Phylogeny of Platelet Molecules and Receptors

One of the first molecules to be investigated phylogenetically has been platelet-derived growth factor (PDGF). The phylogenetic analysis of PDGF was first reported by Singh et al. (1982) who analyzed clotted blood sera from mammals, lower vertebrates, and marine invertebrates to screen for homologues of human PDGF by radioreceptor assay and found that the phylogenetic distribution of PDGF homologues does not correlate with distribution of platelets in evolution (Singh et al. 1982). It was suggested that the coincidental appearance on the vertebrate line of development of one anatomical feature, i.e., a pressurized circulatory system, and of PDGF supports the hypothesis that PDGF plays a role in the maintenance and repair of the vascular lining in vivo. Another related molecule that has been studied is platelet-derived endothelial cell growth factor (PD-ECGF) (Hagiwara et al. 1991). The gene for hPD-ECGF is localized to chromosome 22, and Southern blot analysis using genomic DNAs from several vertebrates suggested that the gene for PD-ECGF is conserved phylogenetically among vertebrates (Hagiwara et al. 1991). Thrombospondin (TSP) is a large, trimeric, modular glycoprotein contained in platelet α-granules (Bornstein 1992) with important roles in cell-extracellular matrix interactions, angiogenesis, synaptogenesis, and connective tissue organization. McKenzie et al. reported that the TSP-1, TSP-3, TSP-4, and TSP-5 genes lie within paralogous regions that provide insight into the ancestral genomic context of vertebrate TSPs. Precursors of the TSP-1 to TSP-5 genes were all present within corresponding ancestral genomic contexts in the last common ancestor of bony fish and tetrapods, and the authors concluded that the TSP-5/COMP protein sequence has evolved to its current state as an innovation of tetrapods and that, given the significant role of TSP-5/COMP in mammalian cartilage, the polypeptide sequence evolved rapidly in tetrapods possibly under the selection pressures imposed on the bony endoskeleton by the switch from aquatic swimming to terrestrial locomotion (McKenzie et al. 2006). Bentley and Adams studied the evolution of TSP and their ligand-binding activities and found that the trimer and pentamer subgroups of vertebrate TSPs represent the outcomes of very different evolutionary events and that the trimerizing coiled coil of TSP-1 and TSP-2 is a chordate innovation. They speculated that the evolution of stable oligomerization by a coiled-coil domain in combination with the increased ligand-binding avidity provided by a trimer was favored strongly under natural selection. The many activities of TSP-1 and TSP-2 that are mediated by their TSR domains in vivo (e.g., TGFβ binding/activation, CD36-dependent anti angiogenesis, and CD36-dependent regulation of nitric oxide-regulated vascular responses) demonstrate the extent to which the functional properties of TSPs were impacted by the evolution of TSP subgroup A (Bentley and Adams 2010). Human platelet antigens (HPAs) are polymorphisms localized on the main platelet receptors (integrin $\alpha_{IIb}\beta_3$, the GPIb-IX-V complex, integrin $\alpha_2\beta_1$) which can stimulate production of alloantibodies in case of transfusion of platelets with different HPAs (Landau and Rosenberg 2011). The HPAs involve single-nucleotide polymorphism at evolutionary variable positions located on the protein surface. These polymorphisms do not cause diseases, but may lead to immunological disorders. Interestingly, some HPAs might also affect the activation process of the protein. The integrins form heterodimers, whereas each subunit comprises multiple domains. This allows a regulation mechanism that involves multiple conformations of the protein on the cell surface (Landau and Rosenberg 2011). Hadhri et al. (2010) studied HPA-1 to HPA-6 and HPA-15 in Tunisian blood donors and found by phylogenetic analysis that Tunisians are more closely related to western (Europeans and North Africans) than to eastern Mediterranean populations. The relationships between North Africans and Mediterranean western Europeans may be explained by the northward Berber migration. It represents a baseline study for future research in the population movement of Maghrebians and may help to better understand the mechanism of platelet alloimmune syndromes.

The $\alpha_{IIb}\beta_3$ receptor is a member of a large family of receptors termed integrins that extend back in evolution to *Drosophila* that are involved in cell adhesion and aggregation, as well as in protein trafficking and bidirectional signaling (Poncz et al. 1987; Fitzgerald et al. 1987; Hynes 1987). Recently, the phylogenetic tree of nine human platelet glycoproteins was reported. Six members (i.e., GPIBA, GPIBB, GP9, GP5, ITGA2, GP2B) were identified as ancestors and showed a close genetic relationship, two (GP3A and GP3B) were close to one another being identified as common GP3, and the last one (CD151) was unique and unrelated to the others (Wiwanitkit 2005) (Fig. 3).

The group of Shaun Coughlin in 1998 compared the amino acid sequences of mouse, human, and *Xenopus* PARs using a computer-based "phylogenetic" analysis program (Pileup) to generate a PAR family tree (Fig. 4) (Kahn et al. 1998).

They speculated that the existence of a *Xenopus* homologue of the PAR1 gene suggests that *Par1g* and *Par2g* diverged before the existence of separate mammalian and amphibian lineages. Considering that all PARs function as thrombin receptors, it is presumable that the capacity to signal in response to thrombin preexisted the divergence of mammals and amphibians. It is possible either that this system played a hemostatic role by mediating platelet or thrombocyte activation in early vertebrates or that thrombin signaling plays a role in embryonic development independently of platelet activation (Connolly et al. 1999), raising the possibility that PARs and signaling in response to thrombin or thrombin-like proteases may have evolved initially to serve a function unrelated to hemostasis (Kahn et al. 1998).

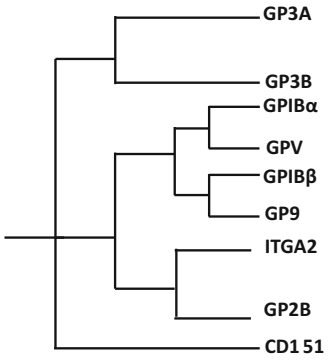

Fig. 3 Phylogenetic tree of human glycoproteins (modified from Wiwanitkit (2005))

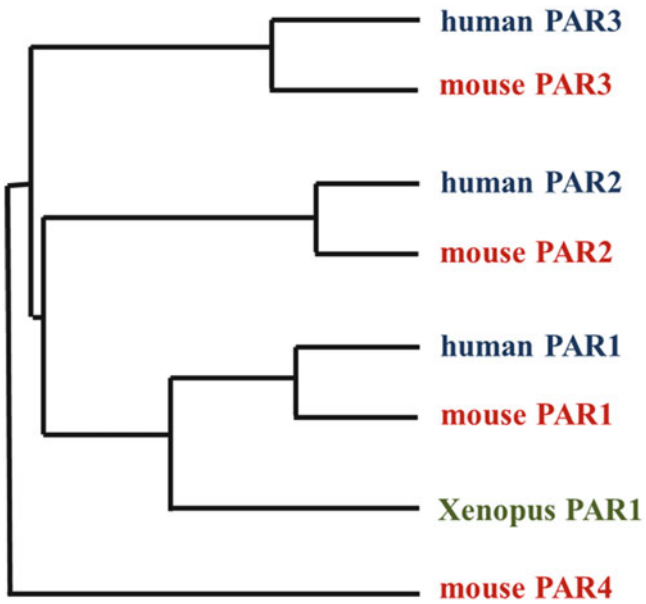

Fig. 4 PAR family tree. Human, mouse, and *Xenopus* genes are shown. Derived from data published on Kahn et al. (1998)

A recent study has compared human platelets and avian thrombocytes. Examination of the genes that are expressed selectively in avian thrombocytes has been carried out by the use of both quantitative PCR and microarray analysis and revealed orthologs for many of the genes known to be required for platelet synthesis and function, including those encoding the MPL receptor that stimulates platelet production; the protease-activated receptors that mediate thrombin activation of platelets; the β_3 and α_2 integrins that mediate platelet adhesion to fibrinogen and collagen, respectively; and the glycoprotein Ib/V/IX (GP9) receptor complex that binds von Willebrand factor (VWF) to mediate adhesion under shear. One notable exception was the lack of thrombocyte-specific upregulation of the gene encoding the ADP receptor P_2Y_{12} in avian thrombocytes, consistently with previous studies showing that they neither contain nor respond strongly to ADP (Schmaier et al. 2011). In addition, chicken thrombocytes spread less efficiently than mammalian platelets on collagen and express low levels of the $\alpha_{IIb}\beta_3$ integrin which is essential for aggregation. The authors suggested that platelets evolved in mammals to provide a hemostatic advantage that in modern humans has become a primary mechanism of cardiovascular disease (Schmaier et al. 2011).

The cyclooxygenase (COX) pathway, which generates prostanoids starting from arachidonic acid, was first reported in 1964 by two independent research teams (see Fries and Grosser 2017). In 1989, the purification of the TP receptor present in human platelets was reported, and in 1991 its cDNA cloned (Fries and Grosser 2017). The TP receptor was identified as a member of the G-protein-coupled rhodopsin-type receptor superfamily. The technique of amino acid sequence comparison has been used to infer the phylogeny of the prostanoid receptors. It was suggested that the ancestral prostanoid receptor responded to PGE2 generated by the COX pathway (Wise and Jones 2002) (Fig. 5). Alignments of all the prostaglandin receptors showed that the EP2 receptor is more similar to the prostacyclin (IP) and the PGD2 (DP) receptors than to the other three EP receptors. Phylogenetic analysis of receptor sequences led to the conclusion that the prostaglandin receptors evolved from a precursor EP receptor into two subfamilies that are characterized by different G-protein coupling. Thus, the IP and DP receptors evolved from the EP2 receptor, and the PGF2α (FP) receptor and thromboxane (TP) receptors evolved from EP3 and EP1 receptor (Fig. 5) (Gill et al. 1998).

Fish thrombocytes convert arachidonic acid to prostaglandins with little, if any, thromboxane formation, whereas thromboxane is a potent inducer of platelet aggregation in mammals (Rowley 1988). Considering the TXA_2/TP receptor relevance in the pathophysiology of the cardiovascular system, evolutionary forces may have favored regulatory mechanisms leading to low basal activity and selected against more highly active phenotypes.

Some distinctive characteristics of platelet function along the phylogenic tree are reported under Table 1.

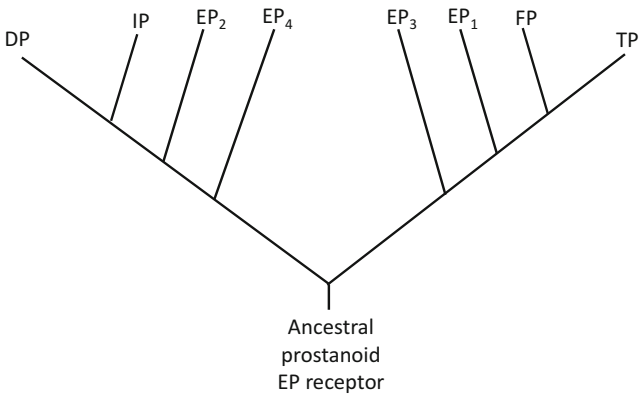

Fig. 5 Proposed evolution of the prostanoid receptors (modified from Wise and Jones (2002))

Table 1 Platelet function characteristics in nonmammalian and mammalians vertebrates as compared to human platelets

	Non mammalian vertebrates	Mammalians									
	Fish, birds, reptiles, amphibians	*Mirounga angustirostris*	*Orcinus orca*	*Equus caballus* (Horse)	*Sus scrofa domesticus* (Pig)	*Ovis aries* (Ovine)	*Canis lupus familiaris* (Dog)	*Rat norvegicus* (Rat)	*Mus musculus* (Mouse) (C57BL/6)	Non human primates	
										Baboon	Cynomolgus monkey
Platelet aggregation											
ADP	Absent (1)	No shape change (2)	Reversible (3)	Aggregate (16)	Reduced (28)	Aggregate (18)	Aggregate (8)	Absent (5,23)	Aggregate (15)	Slightly reduced (34)	Aggregate (34)
Epinephrine	Absent (1)	Reduced (2)	Absent (3)	Aggregate (13)	NA	Reduced (18)	Absent (20)	Aggregate (5)	Absent (16)	NA	NA
Thrombin	Aggregate (9, 11)	No shape change (2)	Reduced (3)	Aggregate (13)	Reduced (28)	Aggregate (26)	Aggregate (20)	Reduced (5)	Aggregate (15)	Aggregate (34)	Aggregate (34)
Collagen	Absent (10) Bird: aggregate (11)	NA	Normal (3)	Aggregate (4)	Aggregate (4)	Reduced (7,8)	Aggregate (8)	Absent (5)	Aggregate (15)	Slightly reduced (34)	Aggregate (34)
PAF	Absent (10) Bird: aggregate (11)	NA	Reversible (3)	Reduced (4)	Reduced (4)	Reduced (4)	Aggregate (7)	Aggregate (5)	NA	NA	Absent (36)
Arachidonic acid	NA	NA	Reversible (3)	Aggregate (4)	Aggregate (19)	Aggregate (19)	Aggregate (19)	Aggregate (22)	Aggregate (15)	Reduced (35)	NA
Ristocetin (agglutination)	NA	Reversible shape change (2)	Absent (3)	Reduced (6)	Absent (24)	Reduced (18)	Absent (20)	Absent (23)	Normal (16)	Normal (34)	Normal (34)
Spreading on fibrinogen	Only in bird (11)	Reduced (2)	Reduced (3)	Reduced (4)	Reduced (4)	Reduced (4)		Absent (5)	Normal (25)	NA	Normal (37)
Thromboxane production	Reduced (11)	NA	Normal (3)	Normal (12)	Normal (27)	NA	Normal (21)	Normal (22)	Normal (15)	NA	Normal (36)
Adhesion on collagen	Reduced (9)	NA		Normal (14, 32)	Normal (32)	Normal (32)	Normal (33)	Reduced (5)	Normal (16)	Normal (38)	NA

(1) Belamarich et al. (1966), (2) Field et al. (2001), (3) Patterson et al. (1993), (4) Pelagalli et al. (2002), (5) Takahashi (2000), (6) Shi et al. (2012), (7) Choi et al. (2010), (8) Sato and Harasaki (2002), (9) Schmaier et al. (2011), (10) Kien et al. (1971), (11) O'Toole et al. (1994), (12) Brainard et al. (2011), (13) Lalko et al. (2003), (14) Watts et al. (2014), (15) Rosenblum et al. (1983), (16) Paigen et al. (1987), (17) Carceller et al. (1996), (18) Tillman et al. (1981), (19) Clemmons et al. (1983), (20) Mischke and Schulze (2004), (21) Schmitz et al. (1985), (22) Ganey and Roth (1987), (23) Takahashi (1991), (24) Zurbano et al. (2000), (25) Morowski et al. (2014), (26) Spanos (1993), (27) Stahl et al. (1997), (28) Kinlough-Rathbone et al. (1993), (29) Daimon et al. (1979), (30) Riboni et al. (1988), (31) Basrur et al. (1983), (32) Pelagalli et al. (2003), (33) Pelagalli et al. (2011), (34) Iwase et al. (2012), (35) van Rensburg 2016, (36) Handley et al. (1986), (37) Cook et al. (1993), (38) Cauwenberghs et al. (2000). *NA* not available

Take-Home Messages

Platelets are unique, highly specialized anucleate mammalian blood cells, with an exclusive molecular repertoire, that have evolved to fulfill crucial functions in host integrity, defense, and repair. However, although they are highly differentiated for hemostasis, platelets also display inflammatory and antimicrobial activities linking the clotting, innate, and adaptive immune responses. This diversity of functions suggests evolution from more primitive multifunctional innate defensive cells, the hemocytes, that mediate wound healing and can induce hemolymph coagulation and clotting at sites of tissue injury or exoskeletal disruption, but that are also involved in host defense against invading pathogens. These primordial hematological responses may point to an evolutionary link between innate immune responses and platelet function. These observations may explain why platelets share many immune-related and tissue-repairing functions, i.e., the functions of the early protective hemocytes that still persist as vestigia in the platelets of mammals. Perhaps, during evolution, the hemostatic and immune functions of hemocytes diverged, and platelets and leukocytes were generated. Studies on the evolution of some platelet proteins confirm a recent development of several platelet-associated molecules further characterizing platelets and their structures as relatively recent specialized actors in the field of host protection.

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Platelet Morphology and Ultrastructure

Harry F.G. Heijnen and Suzanne J.A. Korporaal

Abstract

Platelets are key players in hemostasis, the process that is essential in the prevention of blood loss in response to injury of a blood vessel. When platelets encounter breaches in the vascular wall, they rapidly adhere to the site of injury and aggregate to form a platelet plug. Subsequently coagulation will be initiated resulting in a fibrin network that reinforces the plug. Failure to form an adequate plug leads to a bleeding tendency. On the other hand, excessive platelet reactivity leads to an increased risk of vascular occlusion and thrombosis. In order to better understand how platelets function, it is essential to have insight into their overall morphology and (ultra)structure. This chapter will contribute to this and present our current view of the platelet structure and physiology in health and disease and the recent techniques available to visualize this.

Overall Morphology and Function of Platelets

Blood platelets are small anucleate cell fragments that, together with the red blood cells, are the most abundant cells in the circulation. They derive from megakaryocytes (MKs) in the bone marrow that extend long pseudopodia-like extensions into the sinusoids (Behnke 1969; Italiano et al. 1999, 2007; Thon and Italiano 2010). These extensions, termed proplatelets, further grow due to shear rates of the vasculature and are then thought to fragment to release platelets into the microcirculation. Platelets have a typical discoid shape, a diameter of ~4 μm, and a thickness of ~1 μm. This lentiform shape is brought about by a bundle of peripherally oriented microtubules (White 1968; Italiano et al. 2003) and involves also a complex interplay between the plasma membrane and the underlying spectrin skeleton (Hartwig and DeSisto 1991; Hartwig et al. 1999; Patel-Hett et al. 2011). The discoid shape of the platelets, together with rheological parameters of the flowing blood and the red blood cell shape, ensures that the platelets circulate in a well-defined area in close proximity to the endothelial cell surface (Aarts et al. 1983). These factors are important as the endothelium is the area where the natural inhibitors of platelets (i.e., nitric oxide and prostacyclin) are present at highest concentrations, and it ensures that platelets are able to continuously monitor the endothelium for breaches. Upon endothelial injury, platelets rapidly adhere and interact with components of the subendothelium, i.e., collagen, von Willebrand factor (VWF), and fibronectin, to start restoration of the integrity of the vessel wall (Sixma et al. 1991; Sixma and de Groot 1994). Such interactions generate a series of activation signals in the platelets that result in a dramatic change of their shape, secretion of their granular contents, and the activation of the integrin $\alpha_{IIb}\beta_3$. The latter is the main receptor for fibrinogen and VWF and is responsible for building the hemostatic platelet plug that arrests bleeding after endothelial injury (Fig. 1). Actin-myosin-based platelet retraction finally stabilizes the plug (Hashimoto et al. 1994). Platelet adhesion, aggregation, and secretory responses are the result of a series of complex processes. The unraveling of these processes has been for decades an important goal for researchers in the field of

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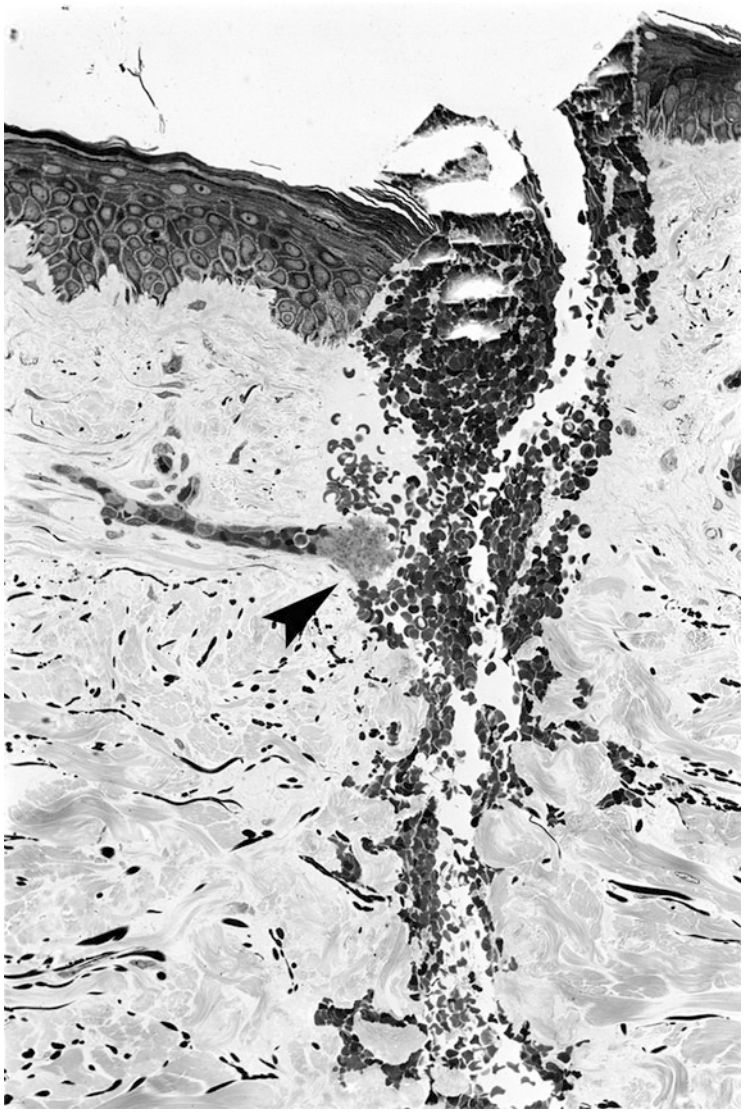

Fig. 1 Hemostasis. Light microscopy of a platelet plug (*arrowhead*) in human skin wound occluding a transectioned blood vessel

hemostasis and thrombosis. Analysis of the platelet ultrastructural changes is key for the understanding of the rapid membrane dynamics that occur during adhesion and activation and has contributed to our present understanding of platelet functioning. In addition, flow devices, real-time imaging, and new developments in light and electron microscopic imaging have offered new opportunities to dissect the relationship between platelet structure and function.

Visualization of Platelets

Platelets can be visualized by light microscopy (LM) on whole blood smears prepared from anticoagulated blood after staining with a May-Grünwaldt Giemsa or Wright stain. In such preparations, platelets appear as small red-bluish, oval and round structures with purple-red puncta as granules. Giant platelet characteristics for macrothrombocytopenia are readily recognized by LM (Zucker-Franklin 2003). This method is therefore suitable for the diagnosis of macrothrombocytopenia-associated platelet disorders and certain storage pool diseases. Platelet adhesive properties can be monitored ex vivo using static adhesion assays or flow devices (Sakariassen et al. 1983; Sixma et al. 1987; Kulkarni et al. 2004). Real-time video microscopy and high-speed CCD cameras coupled to specially developed perfusion devices, and image-processing software, have pushed up both the resolution and sensitivity, making "live" visualization possible of the various steps of platelet adhesion and thrombus building in naturally flowing blood (Fig. 2) and on different physiological surfaces (Fig. 3) (Maxwell et al. 2006; Nesbitt et al. 2013). Confocal microscopy and new super-resolution imaging platforms, such as structured illumination microscopy (SIM), direct stochastic optical reconstruction microscopy (dSTORM), reflection interference contrast microscopy (RICM), and total internal reflection fluorescence (TIRF) microscopy, have provided new ways for imaging of platelet adhesion (Reininger et al. 2006),

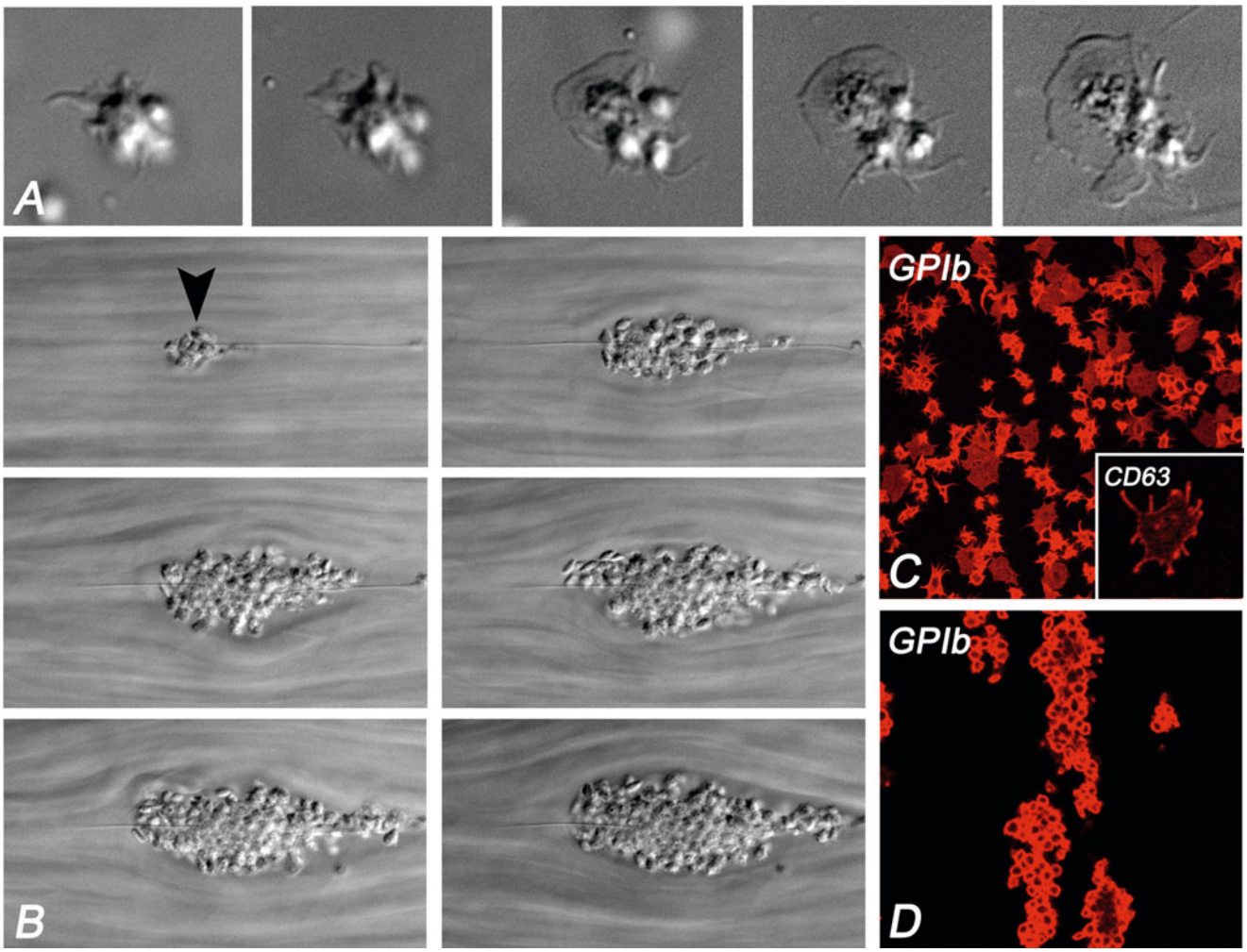

Fig. 2 Real-time and confocal visualization of platelet adhesion. (**a**) Series of still images from video recordings of platelet adhesion and spreading on fibrinogen under flow (DIC), shear rate 100/s. (**b**) Still images taken from live recordings of platelet thrombus formation (*arrow head*) on a collagen surface, shear rate 1600/s. (**c**) Confocal images after whole blood perfusion over fibrinogen, immunolabeling with anti-GPIb or anti-CD63 antibody (*inset*). (**d**) Confocal image after whole blood perfusion over collagen; immunolabeling with anti-GPIb antibody

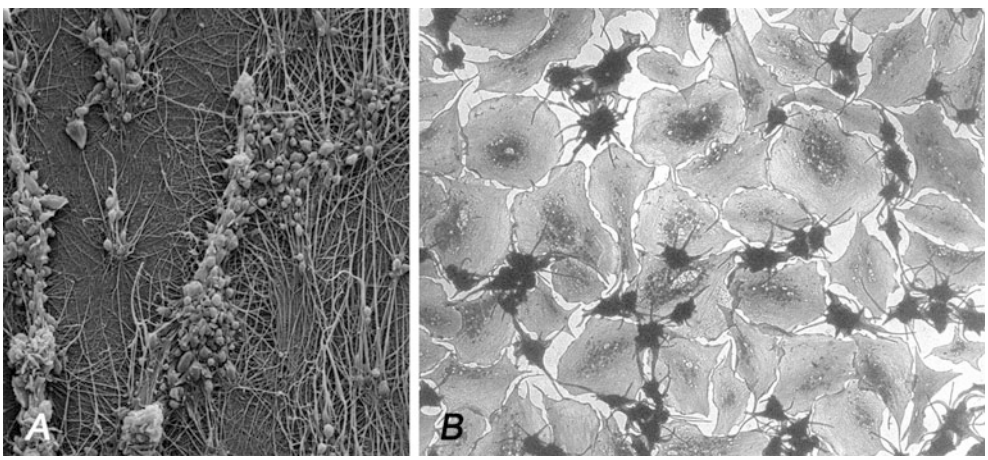

Fig. 3 SEM and TEM imaging of platelet adhesion. (**a**) Scanning electron microscopy of platelet thrombus formation and fibrin formation, following interaction under flow with endothelial cell matrix. (**b**) Whole-mount transmission electron microscopy of platelet adhesion and spreading on fibrinogen under flow

Ca(2+) dynamics (Nesbitt et al. 2003), cytoskeletal rearrangements (Poulter et al. 2015), as well as molecular localization and translocation events (secretion) (Fig. 2c, inset) (Kamykowski et al. 2011). In addition, fluorescently labeled phalloidin, organelle-specific fluorescent dyes (e.g., mepacrine or acridine orange), specific fluorescent calcium indicators, and cell surface and secretion markers are now widely used to study platelet adhesion dynamics (Fig. 2c, d).

Electron microscopy (EM) has always played an important role in the various branches of platelet research. Scanning electron microscope (SEM) has been widely used for obtaining images of the platelet surface topology at rest or after interaction with physiological substrates (Fig. 3a). Such SEM preparations provide information on functional alterations such as shape change, the size of thrombi, and the formation of fibrin. Whole-mount preparations of platelets adhered to physiological surfaces such as fibrinogen, VWF, or collagen can also be prepared on substrate-coated EM grids and studied by transmission electron microscopy (TEM) after uranyl staining and embedding in methylcellulose (Fig. 3b). Combined with immunogold labeling strategies, these techniques provide information on the topographical distribution of cell surface receptors and associated ligands (Lewis et al. 1990; Beumer et al. 1995).

Thin section TEM is the most widely used method to visualize platelet ultrastructure (Fig. 4). With TEM, the intracellular organization of organelles and to some extent

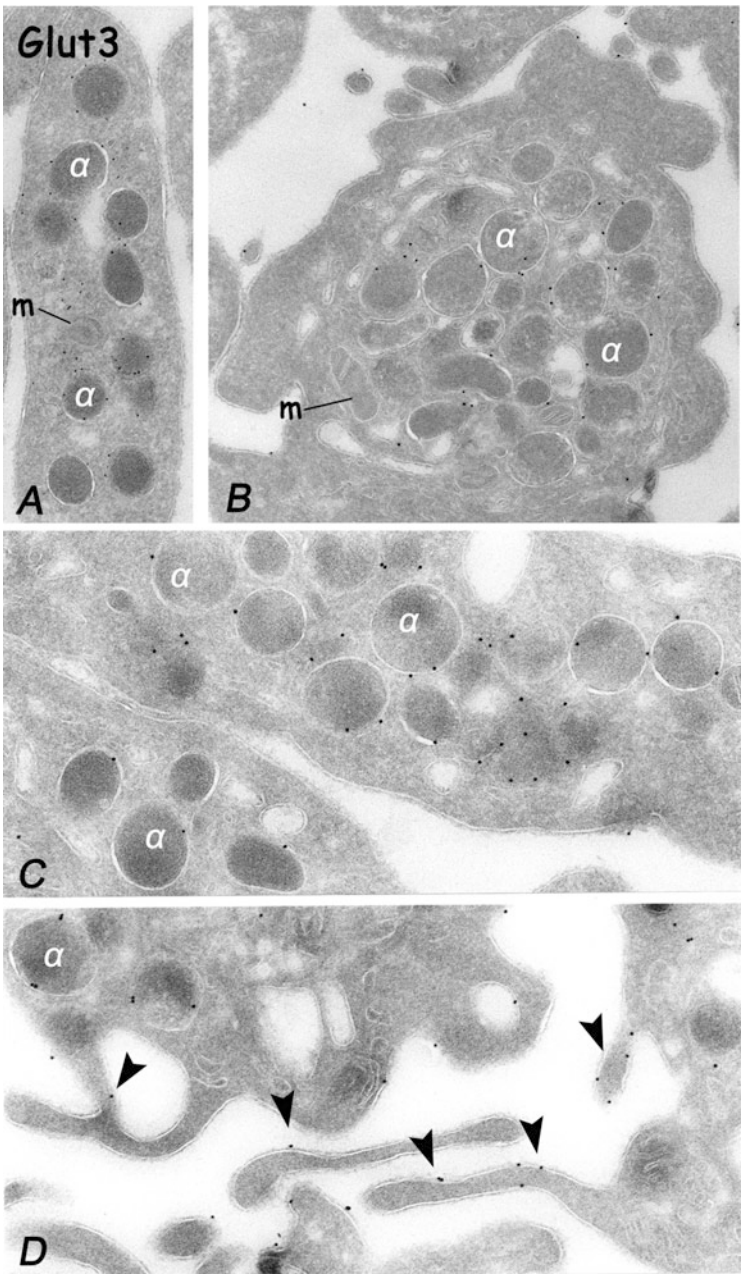

Fig. 4 TEM of resting and stimulated platelets. (**a**) Thin frozen section of a discoid platelet obtained from whole blood directly drawn into fixative. Immunolabeling with an antibody directed to glucose transporter Glut3. (**b**) Loss of discoid shape, formation of filopodia, and centralization of alpha granules after activation with ADP. (**c**, **d**) Thrombin stimulation induces secretion of alpha granule content and is accompanied by translocation of alpha granule membrane proteins (Glut3) to OCS and cell surface (*arrowheads*). *α* Alpha granules, *m* mitochondria

also cytoskeletal elements can be visualized. The classical method includes chemical fixation using aldehydes, dehydration in a series of graded dilutions of alcohol, and embedding in an epoxy resin. Ultrathin sections are prepared and visualized by TEM following heavy metal staining. Such TEM preparations provide only 2D images of the cellular ultrastructure and limited information within the Z-axis. In addition, the harsh fixation protocols involving OsO4, and embedding in epoxy resins, limit antibody access and thereby detection of the subcellular distribution of proteins. New technologies have been developed that overcome these limitations. These include the use of cryo-ultramicrotomy and immunogold labeling of frozen thin sections (i.e., the Tokuyasu method) (Fig. 4) (Tokuyasu 1973; Geuze et al. 1979; Morgenstern 1980) and the generation of new (3D) electron microscopic techniques to image larger cell volumes (Fig. 5) (van Nispen tot Pannerden et al. 2010; Eckly et al. 2014). Although chemical fixation does not arrest rapid membrane dynamics, it is still the most commonly used method to preserve platelets for ultrastructural analysis and immunogold cytochemistry. Immuno-electron microscopy is increasingly becoming a routine technique to precisely localize molecules within their subcellular context, enabling visualization of protein transport routes and molecular translocation events (Fig. 4c, d) (Heijnen et al. 1997, 1998). In addition, cryo-immobilization methods (high-pressure freezing (HPF)) and electron tomography approaches (dual-axis tomography, focused ion beam/scanning electron microscopy (FIB/SEM)) are more and more entering the field and provide the opportunity to visualize the spatial 3D organization of cellular structures (van Nispen tot Pannerden et al. 2010; Eckly et al. 2014).

The Resting Platelet Ultrastructure

Platelets contain a number of structural elements, most of which have been characterized by traditional TEM (Behnke 1967a, 1968a, 1970; White 1968, 1969; White and Clawson 1980; White and Krumwiede 2007). They include the plasma membrane (PM); the open canalicular system (OCS), an elaborate membrane system that is contiguous with the plasma membrane; the dense tubular system (DTS), a reticular membrane network, and remnant of the smooth endoplasmic reticulum (ER); a peripheral band of microtubules; a spectrin-based membrane skeleton; an actin-based cytoskeletal network; and a population of cytoplasmic organelles that include mitochondria, peroxisomes, and secretory alpha granules, dense granules, and lysosomes. Platelets are very vulnerable to activation. Contact activation during blood collection and handling steps during isolation and centrifugation may cause subtle pre-activation, resulting in premature loss of the discoid shape and possibly secretion of granule content. Hence, for ultrastructure

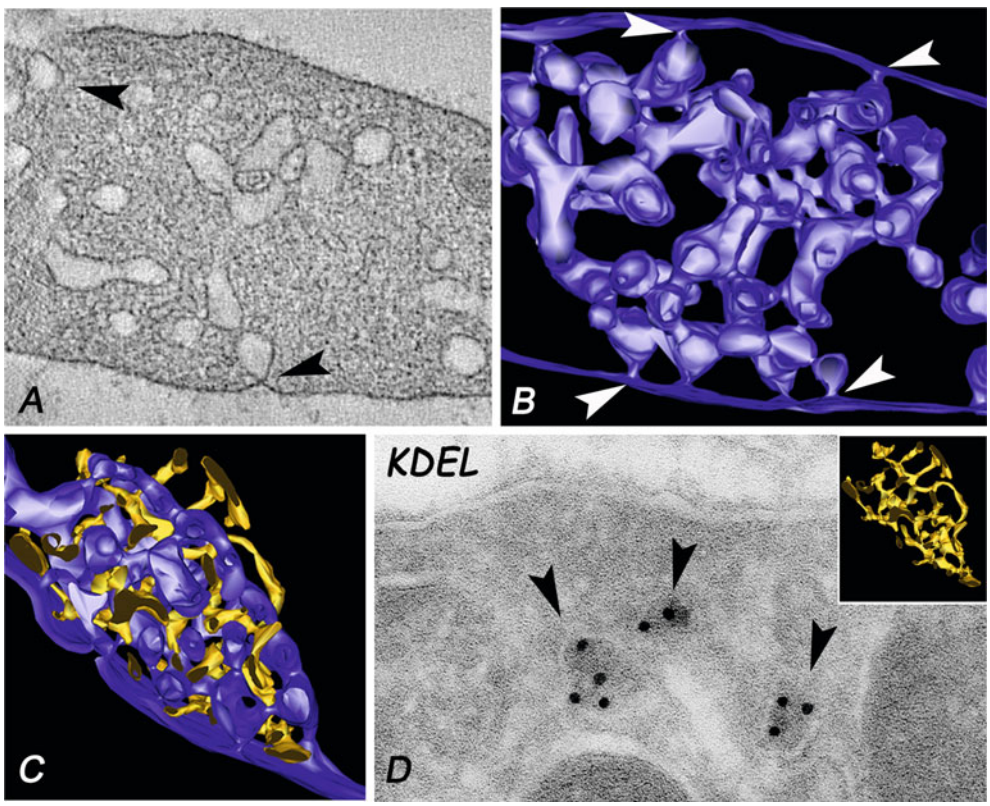

Fig. 5 Open canalicular system (OCS) and dense tubular system (DTS). Tomographic slice (**a**) and 3D reconstruction (**b**) of OCS showing multiple cell surface connections (*arrowheads*). (**c**) Closely intertwined OCS (*blue*) and DTS (*yellow*) membranes. (**d**) Identification of the DTS using immunogold labeling with an antibody directed to the ER retention motif KDEL. The electron-dense structures represent cross-sectioned membranes of the reticular DTS shown in the *inset*

analysis of the steady-state resting platelet, blood handling should be kept at a minimum. Immediate chemical fixation by collecting whole blood directly from the vein into excess aldehyde fixative is a well-recommended method to preserve the platelet resting state. Alternatively, whole blood can be drawn into citrate, followed immediately by aldehyde fixation. The platelet isolation steps can then be performed after fixation.

The Open Canalicular System

Platelets harbor an elaborate internal membrane system, termed the open canalicular system, based on the fact that it is connected at several points to the cell surface via small ~20 nm openings (Fig. 5a, b) (Behnke 1970; White and Clawson 1980). The OCS probably derives from the demarcation membrane system, a highly intertwined membrane network which develops in the MK and ultimately forms the future platelets (Behnke 1968b; Eckly et al. 2014). Although it is not considered a separate intracellular compartment, the OCS still represents an important internal membrane store with specific functions. It facilitates membrane remodeling and shape changes that occur during platelet adhesion, including filopodia formation, membrane tethering, and irreversible spreading. The increase in membrane needed for these morphological changes is believed to be provided by the OCS (Escolar et al. 1989), as well as by membranes of the secretory alpha granules (Peters et al. 2012). Whether internal OCS membranes are able to fuse with the PM, which represents the same membrane, is currently not known. It is generally thought that OCS membranes evaginate when platelets spread on physiological relevant surfaces such as VWF or fibrinogen (Escolar et al. 1989). Platelets make use of membrane-based tethers to transiently adhere to VWF under high shear conditions (Dopheide et al. 2002; Maxwell et al. 2006; Reininger et al. 2006). These membrane tethers, which can reach a length up to 50 μm, are formed when biomechanical forces generated by the blood flow act on GPIb–VWF bonds, thereby pulling membrane from internal platelet stores. Since these transient interactions do not require platelet activation, the membrane must exclusively derive from the OCS. Long membrane extensions are also formed as a combined result of platelet activation and shear forces (Cranmer et al. 2011; Tersteeg et al. 2014). Recent studies have confirmed that a link exists between the structure of the OCS and shear-dependent platelet adhesive function (Mountford et al. 2015). The dynamic nature of the OCS may explain the rapid redistribution of cell surface GPIb/IX from the cell surface toward the OCS, as proposed by some investigators (Hourdille et al. 1990; Han et al. 2003), although this has been challenged by others (White et al. 1996). Receptor redistribution may serve to regulate platelet adhesive properties (van Zanten et al. 1998).

Since the OCS has multiple openings to the plasma membrane (Fig. 5a, b), it provides a two-way route for both plasma components to "enter" the cell (Klement et al. 2009), as well as serving as a conduit for cargo secretion from alpha and dense granules (White 1987; Escolar et al. 1989). OCS membranes typically have the same cell surface distribution as the PM and are highly intertwined with the DTS (Fig. 5c) (van Nispen tot Pannerden et al. 2010). Proper identification of the OCS and its distinction from other membrane systems are essential. Early studies have made use of ruthenium red to specifically stain the OCS (Behnke 1968a). In thin frozen sections, identification of the OCS, especially its distinction from the DTS, requires specific cell surface marker antibodies (i.e., GPIb) or DTS marker antibodies (KDEL, PDI; Fig. 5d).

The Dense Tubular System

Except for the small population of young reticulated platelets, circulating platelets have no ribosomal ER. Instead, platelets contain a dense tubular system (DTS), which is a smooth endomembrane system that originates from the endoplasmic reticulum (ER) of the megakaryocyte (White 1972). The DTS stores and releases calcium in a fashion similar as the sarcoplasmic reticulum of the skeletal muscle. The DTS also harbors a series of ER enzymes that are important for the proper folding of proteins, like PDI and calreticulin. Early ultrastructural studies have demonstrated that the DTS also contains peroxidase activity, which allows specific staining in plastic sections (Breton-Gorius and Guichard 1972). When visualized in thin frozen sections, DTS membranes appear as small tubulevesicular membrane structures, with frequently an electron-dense appearance (Fig. 5d, arrowheads). This has prompted investigators to term these structures T-granules, given their tubular morphology (Thon et al. 2012). T-granules contain TLR9, PDI, and VAMP-8, and it has been suggested that these granules are recruited to the cell surface, thereby contributing to protein secretion, e.g., the secretion of PDI. Extracellular PDI released from activated platelets (Chen et al. 1995) has been shown to contribute to platelet thrombus formation (Cho et al. 2008, 2012; Furie and Flaumenhaft 2014; Schulman et al. 2016). A direct fusion step, however, of DTS membranes with the OCS or plasma membrane has so far not been established. Early work (White 1972) and recent electron tomography analysis have shown that the DTS represents a reticular membrane network (Fig. 5d, inset), frequently found in close vicinity of the OCS, suggesting functional communication between both compartments (van Nispen tot Pannerden et al. 2010).

Platelet secretion and contraction-dependent shape changes require a rapid increase in cytosolic calcium derived from internal stores (i.e., DTS). The close positioning of both membrane systems to one another foresees in local calcium delivery required for SNARE assembly as well as actin-dependent retraction.

Peroxisomes

Peroxisomes are single-membrane organelles that morphologically range from rather small vesicles in some cells to elaborate tubular networks in others (De Duve and Baudhuin 1966). Peroxisomes are thought to derive from the ER (Tabak et al. 2003). In platelets they are very small organelles and are probably closely connected to the DTS. Peroxisomes contribute to lipid metabolism, in particular the synthesis of platelet-activating factor (PAF). Deficiencies of this enzymatic activity in platelets have been identified in the cerebro-renal Zellweger syndrome (Wanders et al. 1984). A consistent feature of peroxisomes is the presence of H_2O_2-producing oxidases and catalase degrading H_2O_2. Platelet peroxisomes were identified through the presence of catalase activity using a diaminobenzidine reaction at pH 9.7 and visualization by EM (Breton-Gorius and Guichard 1972).

Mitochondria

Platelets contain on average seven mitochondria (Morgenstern 1997), which are involved in oxidative energy metabolism (Akkerman and Holmsen 1981). Platelet mitochondria have a diameter of approximately 200 nm and are easily identified by their classical double membrane structure with inward membrane projections forming the cristae (Fig. 4a, b) (Alberts et al. 2002). As in other cells, platelet mitochondria contain the typical activity of oxidative phosphorylation and respiratory control to provide ATP for regulation of platelet function. Together with glucose entry, mitochondria contribute to maintain a sufficient platelet energy content, both at steady state and during platelet activities such as adhesion, aggregation, secretion, and contraction (Garcia-Souza and Oliveira 2014). Mitochondria are particularly important in metabolically active tissues such as the skeletal muscle, heart, and liver. It is becoming more and more evident that mitochondria not only regulate activation of the metabolically active platelets by providing ATP. They also regulate intracellular signaling through the production of reactive oxygen species (ROS) as a second messenger (Zharikov and Shiva 2013). Furthermore, mitochondria initiate apoptosis through the release of cytochrome *c* (Lopez et al. 2007), which is important for the survival of platelets. An interesting aspect recently documented is that activated platelets can release their mitochondria in the form of microparticles (Zharikov and Shiva 2013).

α-Granules

α-Granules are the most abundant secretory organelles in platelets (50–80 per platelet). They constitute about 10 % of the total platelet volume, and their total membrane surface area equals that of the OCS (Morgenstern 1997). α-Granules contain a large variety of adhesive proteins that are important for primary hemostasis, including the adhesive proteins VWF, fibrinogen, fibronectin, vitronectin, and thrombospondin (Fig. 6) (Sander et al. 1983; Wencel-Drake et al. 1985, 1986). These proteins are important for platelet adhesive properties and the building of a stable thrombus. Platelet α-granules also contain a large number of mediators that function in coagulation, wound repair, inflammation, and angiogenesis. These include among others platelet factor 4 (PF4), β-thromboglobulin (CXCL7), Rantes (CCL5), interleukin-8 (IL-8), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF) (Maynard et al. 2007). PF4, β-thromboglobulin, and Rantes are the most abundant chemokines in platelet α-granules (Deuel et al. 1981; Labelle et al. 2014). α-Granules acquire cargo from biosynthesis (e.g., VWF, β-thromboglobulin, PF4) as well as from endocytosis (e.g., fibrinogen, albumin, IgG) (Harrison et al. 1990). α-Granules thus have properties of both secretory organelles and late endosomes. Indeed, α-granules frequently harbor clathrin coats and luminal vesicles (Fig. 6a, a′), a tribute that is shared with sorting compartments such as the trans-Golgi network and endosomes. The classical view that platelet α-granules represent a homogeneous population of organelles has been challenged by several groups. Italiano et al. reported that anti-angiogenic factors, such as endostatin, reside in a different granule population than proangiogenic VEGF (Italiano et al. 2008). Such subpopulations of α-granules were suggested to respond to specific agonists and hence release granule contents in a thematic fashion (Chatterjee et al. 2011). Early ultrastructural studies (Cramer et al. 1985; Harrison et al. 1990) and recent electron tomography analysis (van Nispen tot Pannerden et al. 2010) have shown that cargo is heterogeneously distributed within α-granules and that morphologically different classes of α-granules exist (Fig. 6). While a majority of the α-granules have a spherical shape, tubular and multivesicular subtypes are also frequently identified (van Nispen tot Pannerden et al. 2010). These studies and recent quantitative super-resolution immunofluorescence microscopy have further established that cargo proteins within individual α-granules are spatially segregated,

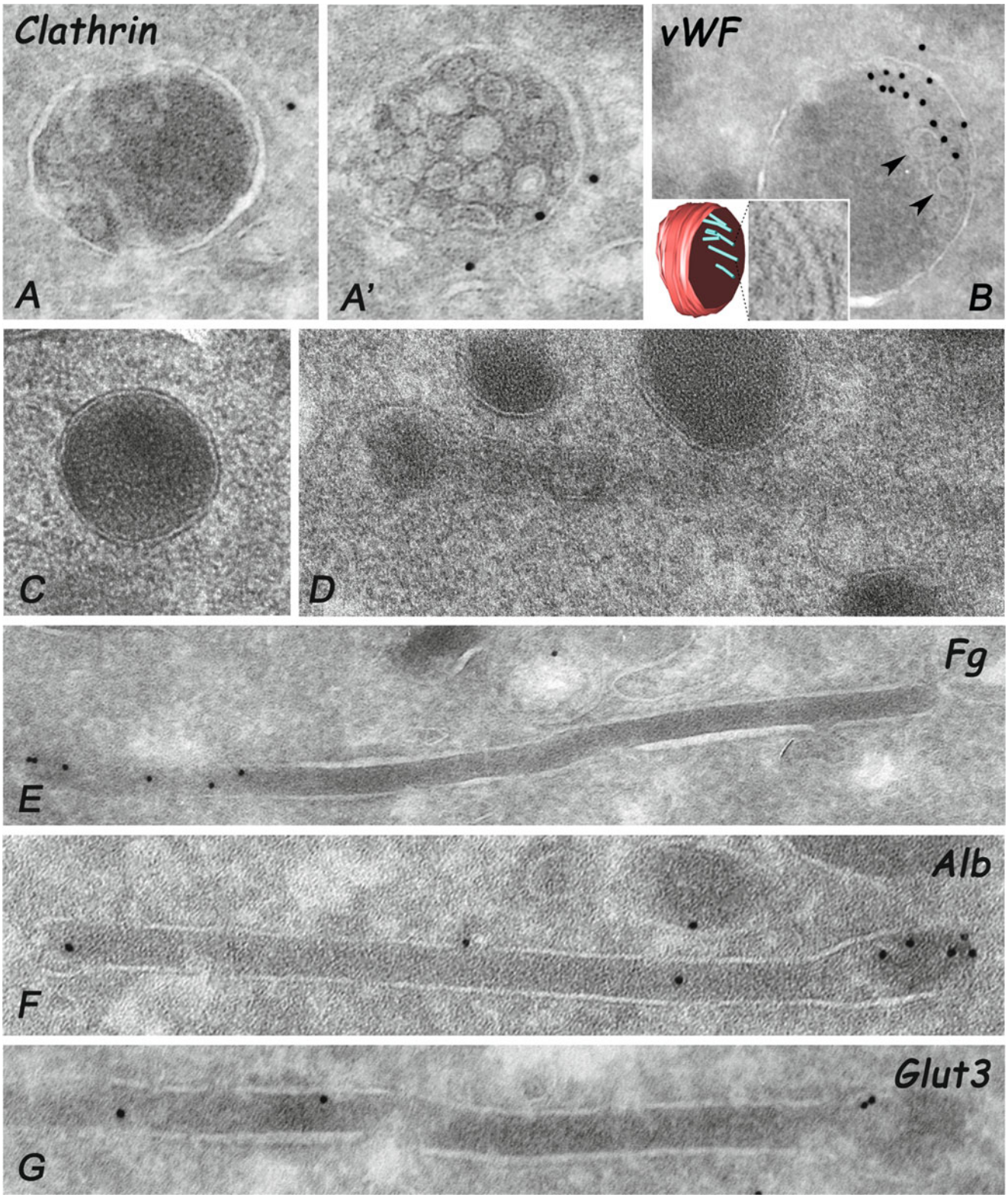

Fig. 6 Alpha granule substructure. (**a**, **a**′) Two subsequent sections through the same alpha granule containing electron-dense cargo and luminal vesicles (immunogold labeling with anti-clathrin antibody). (**b**) vWF multimeric protein assembled at the granule periphery (immunogold labeling with anti-vWF). *Arrowheads* indicate luminal vesicles. The *inset* shows a 3D reconstruction (*left*) and high magnification (*right*) of the tubular vWF inclusions. (**c**, **d**) Spherical- and tubular-shaped granules in high-pressure frozen resting platelets. (**e**–**g**) Tubular alpha granules with spatially distributed cargos including fibrinogen and albumin and the presence of the transmembrane transporter protein Glut3

apparently without much thematic selectivity (Kamykowski et al. 2011). Large cargo proteins (i.e., VWF) are thought to be sorted into secretory granules through aggregation. Recent studies in endothelial cells have provided a model by which clusters of VWF multimers aggregate and tubulate to form the Weibel-Palade bodies (WPBs) (Mourik et al. 2015). As in WPBs, multimeric VWF in α-granules is also assembled in distinct tubular structures and segregated from other molecules (Fig. 6b, inset) (Cramer et al. 1988). An important question is to what extent diverse cargo is released with different kinetics and how this is regulated, considering that these molecules originate from the same granule. Release of thematically different cargos could stem from the dilution of the protein gradient within the compartment once the granule membrane has fused with the PM or OCS or rely on specific signaling pathways and/or sorting of SNARE components (Flaumenhaft 2003; Peters et al. 2012). Early studies using cryo-fixation methods have shown transient fusion pores of α- and dense granules with the PM (Hols et al. 1985; Morgenstern et al. 1987). Such fusion pores may deliver small molecules, while the larger proteins are retained.

Patients with defects in platelet α-granule biogenesis have mild to moderate bleeding problems. The defects are mostly caused by mutations in the genes encoding neurobeachin-like 2 (NBEAL2) and the Sec1/Munc18 protein VPS33B and its binding partner VPS16B, which are required for biogenesis of α-granules. Mutations in *NBEAL2* cause gray platelet syndrome (GPS), a hereditary, usually autosomal recessive bleeding disorder, characterized by macrothrombocytopenia and specific deficiency of α-granules and/or their content (Albers et al. 2011; Gunay-Aygun et al. 2011; Kahr et al. 2011). The severity of GPS depends on the extent of α-granule deficiency (Bottega et al. 2013). GPS platelets appear pale gray when visualized by LM in whole blood smears, due to the absence of the cytoplasmic puncta representing the α-granules. EM of thin sections of platelets from GPS patients shows only vestigial α-granules and abundant channels of the OCS (Fig. 7). Recently, a nonsense mutation in the gene encoding the transcription factor growth factor independent 1B (GFI1B) was shown to cause an autosomal dominant form of GPS (Monteferrario et al. 2014).

Both VPS33B and its binding partner VPS16B are essential for the biogenesis of platelet α-granules. Mutations in both proteins result in arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome that, similar to GPS, is characterized by pale-appearing platelets in whole blood smears with loss of α-granules (Urban et al. 2012).

Dense Granules

Platelet dense granules (3–8 per platelet) are the second major secretory compartment. They are mostly spherical in nature (~200 nm) but can also adopt extended forms (Fig. 8). Based on their high nucleotide content, dense granules possess a high affinity for typical nuclear stains (DAPI) and uranium ions (Richards and Da Prada 1977). Dense granules belong to the same class of organelles as acidocalcisomes (Docampo et al. 2005), which due to their acidic nature accumulate acidophilic dyes such as acridine orange and mepacrine. Dense granules contain small molecules such as ADP, ATP, serotonin, calcium, pyrophosphate, and polyphosphate (Ruiz et al. 2004). The low molecular mass content makes dense granules difficult to visualize by EM. In frozen thin sections, the dense core is often partly or completely lost, leaving only the limiting membrane, and their identification requires labeling with specific membrane

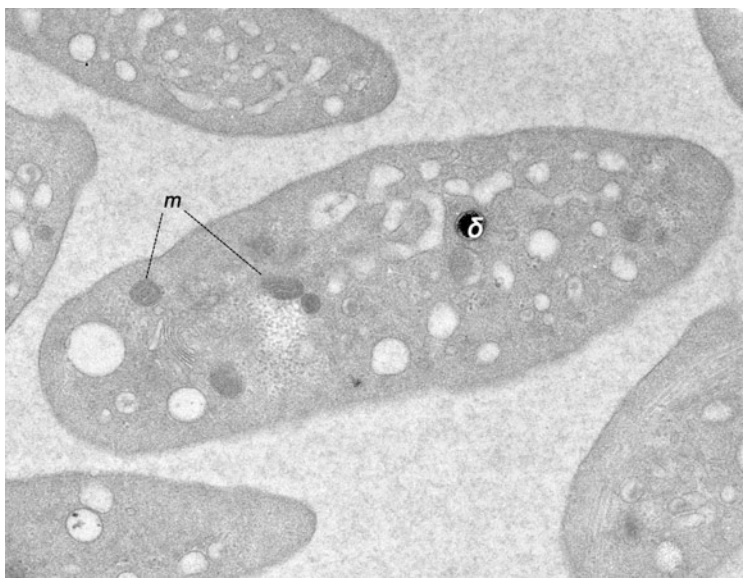

Fig. 7 Gray platelet syndrome. TEM image of platelet from a patient with gray platelet syndrome, showing lack of alpha granules. *m* Mitochondria, *δ* dense granule

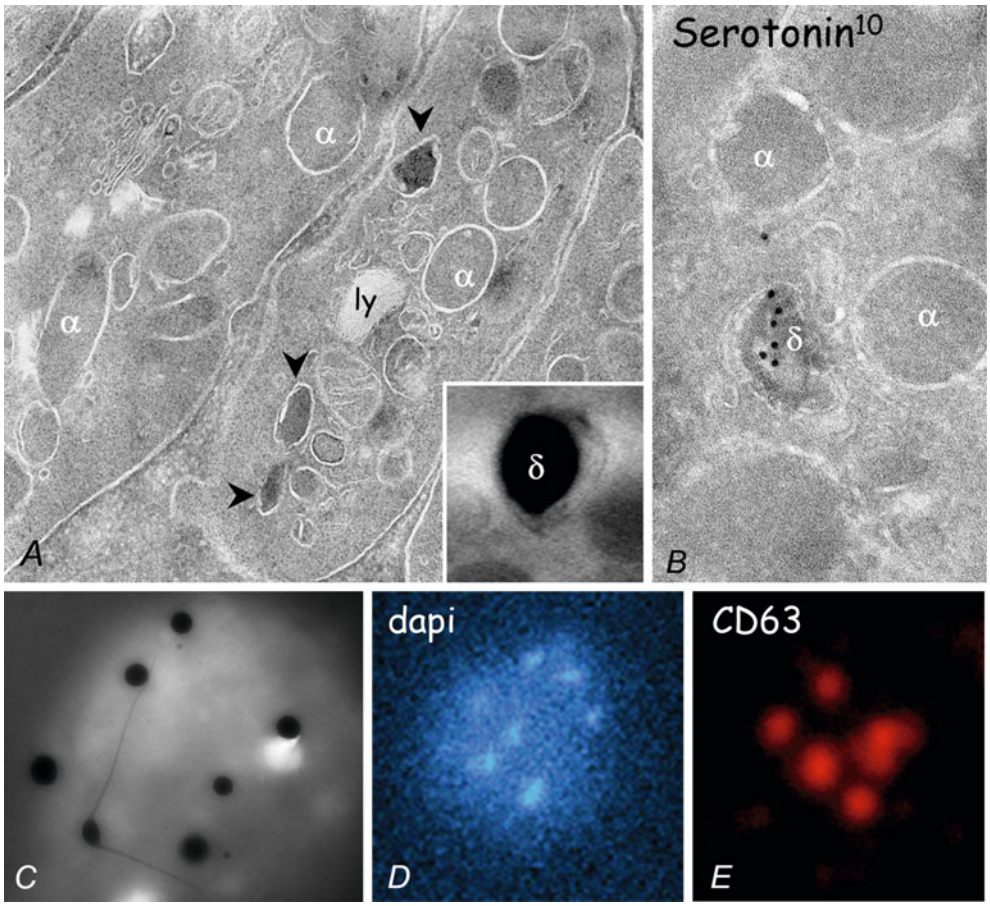

Fig. 8 Visualization of dense granules. (**a**) Uranaffin reaction on thin frozen sections showing dense granules (*arrowheads*). *Inset*, slice from a cryo-tomogram. (**b**) Immunogold labeling of serotonin in thin frozen section. (**c**) Whole-mount TEM visualization, showing the electron-opaque dense granules. (**d**, **e**) Immunofluorescence images of dense granules. (**d**) DAPI staining. (**e**) Immunostaining with monoclonal anti-CD63 antibody. *δ* Dense granules, *α* alpha granules, *ly* lysosomes

or content markers (Fig. 8b). Dense granules contain the lysosomal membrane proteins CD63 and LAMP1/LAMP2 (Israels et al. 1992), which become expressed on the cell surface after activation. Translocation of these lysosomal markers to the cell surface can be monitored by FACS analysis or IF and may represent a functional readout for the identification of delta-storage pool deficiencies. Dense granules originate from endosomal precursors rather than from the trans-Golgi network (Meng et al. 2012) and play a crucial role in primary hemostasis by stimulating the platelet P2Y12 receptor after release of ADP, thereby initiating a feedback platelet activation cycle. The predominant presence of calcium and (poly)phosphates is the reason for their electron-opaque appearance in whole-mount EM and cryo-EM preparations (Fig. 8a, c, inset). This feature is lacking in the Hermansky-Pudlak (HPS) and Chediak-Higashi syndromes (CHS), two delta-storage pool disorders characterized by the absence or deficiency of dense granules. Whole-mount EM analysis represents a classic diagnostic tool for such disorders (Al Hawas et al. 2012). HPS is a rare autosomal recessive disorder caused by defects in any of nine genes, resulting in different subtypes of the syndrome, characterized by a defect in the secondary platelet response, oculocutaneous albinism, and life-threatening pulmonary fibrosis (Huizing et al. 2008). CHS is an autosomal recessive immunodeficiency disorder caused by mutations in the *LYST* or *CHS1* gene that affect the synthesis and/or maintenance of storage/secretory granules in various types of cells.

Lysosomes

Platelet lysosomes and residual bodies have been described in early immunocytochemistry studies (Bentfeld-Barker and Bainton 1982; Sixma et al. 1985; Behnke 1992). Platelet lysosomes contain acid hydrolases (e.g., cathepsins, hexosaminidase, β-galactosidase, arylsulfatase, β-glucuronidase, and acid phosphatase) as most important cargo, and similar as dense granules, they express CD63 and LAMP1/LAMP2 (Metzelaar et al. 1991). Lysosomes serve a role in the digestion of phagocytic and cytosolic components, similar to their roles in nucleated cells (Behnke 1992), and participate in the platelet secretory response (Holmsen and Day 1968). Secretion of lysosomal content may have several important extracellular functions such as supporting receptor cleavage, assisting fibrinolysis, and degradation of extracellular matrix components. Platelets have a core autophagy machinery, which is constitutively active and becomes upregulated

under starvation conditions and after activation (Feng et al. 2014; Ouseph et al. 2015). The cytosolic substrates that are target for autophagic sequestration in platelets are unknown. Platelet lysosomes may contribute to a process termed unconventional secretion, whereby selective cytosolic material is secreted (Nickel and Rabouille 2009). Recent studies have shown that platelets synthesize proteins (e.g., tissue factor, IL-1β, Bcl-3), which are released upon activation (Schwertz et al. 2006; Zimmerman and Weyrich 2008). The cytosolic nature of these newly synthesized proteins requires a special kind of secretory mechanism. Microvesicles, exosomes, and/or selective autophagic targeting and (auto)-lysosomal secretion may provide a way for these cytosolic proteins to exit the cell.

Platelet Cytoskeleton

Platelets contain three types of cytoskeletal elements: microtubules, the membrane skeleton, and the actin cytoskeleton. Microtubules in platelets are arranged in a marginal bundle at the cell periphery. Insights into the cytoskeleton and marginal band structure have come from early microscopic studies (Behnke 1967b; Behnke and Zelander 1967; Zucker-Franklin 1969; White 1982; White and Rao 1998). In contrast to the usual rigidity of microtubules in other cells, the platelet marginal band is remarkable flexible. β1-Tubulin, the major isoform, and unique for MKs and platelets, is the most important component of the microtubular coil and is thought to give the coil its flexibility and typical structural array, thereby maintaining the elliptical shape of the circulating platelets (Fig. 9). Mice lacking β1-tubulin have spherical platelets and a defective marginal band containing only 2–3 microtubule coils (Schwer et al. 2001; Italiano et al. 2003). Platelet microtubules are sensitive to environmental conditions. Platelet exposure to 4 °C induces a loss of the discoid shape (spherocytosis) due to disassembly of the microtubules (White 1982; White and Rao 1998). The marginal band in platelets has long been thought to be composed of a single microtubule, coiled at the platelet periphery. However, a recent study showed that it consists of a stable microtubular coil, with multiple associated, short dynamic microtubules and with both plus and minus ends arranged along the coil (Patel-Hett et al. 2008). Dynein-dependent sliding of microtubules, elongation, and MT band coiling induces the spherical shape during early platelet activation,

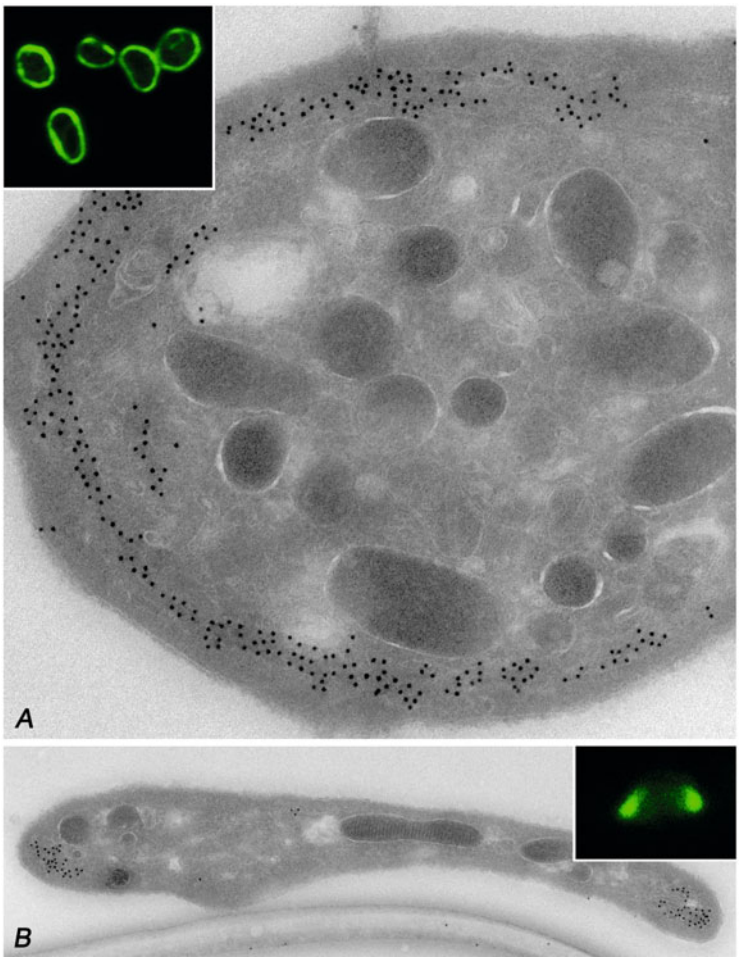

Fig. 9 Peripheral microtubular coil. Microtubules in platelets are arranged in a marginal bundle at the cell periphery. Immunofluorescence (*inset*) and immunogold staining of resting platelets using alpha-tubulin antibody. Staining patterns are shown in the equatorial (**a**) and cross-sectional plane (**b**)

whereby newly polymerizing microtubules in concert with actin-myosin tension (see below) will form a smaller microtubular ring (Diagouraga et al. 2014; Sadoul 2014).

The platelet cytoskeleton consists of a spectrin-rich membrane skeleton lining the inner plasma membrane and a network of long actin filaments that radiate from the cell center to the surface membrane (Fox et al. 1988). The percentage of filamentous actin rapidly increases upon platelet activation (Hartwig 1992, 1999), which, through actin-binding proteins, introduces a link of the cytoskeleton with surface membrane glycoproteins (Fox 1985). The membrane skeleton is essential for maintaining the structure and integrity of the surface membrane, whereas the cytoskeleton generates contractile forces within the cell, thereby regulating platelet shape change and granule dynamics during secretion (Painter and Ginsberg 1984; Flaumenhaft 2003). Early in vitro observations have shown that platelet secretion is accompanied by granule centralization, implicating actin-myosin dependent contractile forces facilitating the release of granule contents (Painter and Ginsberg 1984; White et al. 1984a; Flaumenhaft 2003). The exact mechanism, however, by which reorganization of the actin cytoskeleton affects granule fusion events remains poorly understood. Cortical actin has been reported to provide both a natural barrier as well as playing a facilitative role in regulated exocytosis in other nucleated cells (Orci et al. 1972; Muallem et al. 1995; Trifaró et al. 2008). Cytochalasins and latrunculin-A have been shown to increase cargo release from dense granules, indicating that actin depolymerization promotes dense granule exocytosis (Flaumenhaft et al. 2005; Ge et al. 2012). Actin and myosin are also required for the overall process of clot retraction (Zucker-Franklin and Grusky 1972; White and Burris 1984; Morgenstern et al. 1984, 1990), a process that is essential for stabilization of the thrombus. Specific whole-mount methods using detergent extraction and stabilization of the actin cytoskeleton by shadow-casting, freeze fracture methods, or IF staining of phalloidin have been developed to study the organization of the platelet cytoskeleton during adhesion and spreading (Hartwig and DeSisto 1991; Hartwig 1992; Poulter et al. 2015). Using a combination of EM and high-resolution IF microscopy approaches, two novel actin structures, podosomes and actin nodules, were recently characterized in megakaryocytes and platelets (Schachtner et al. 2013; Poulter et al. 2015). Actin nodules are linked by multiple actin bundles and play a key role in the spatial localization of adhesion-related proteins, e.g., integrins. Podosomes are actin-rich cytoskeletal elements required to degrade basement membranes in megakaryocytes, possibly enabling proplatelets to reach the sinusoids and form proper platelets. Loss of podosome formation has been suggested to contribute to the reduced platelet number, a smaller size of platelets, and lower granule number, observed in patients with Wiskott-Aldrich syndrome (WAS), an X-linked recessive disease (Schachtner et al. 2013; Poulter et al. 2015). Actin nodules can be monitored on adherent platelets using specific antibodies directed to actin, WASP, or ARP2/ARP3.

Platelet Microparticles (PMPs) and Exosomes

Platelet adhesion and activation is accompanied by the release of membrane-bound vesicles. The small-sized population (40–100 nm) of these membranes is secreted from the multivesicular granule population and is termed exosomes (Heijnen et al. 1999). Platelet-derived exosomes are enriched in CD63, a tetraspanin protein also found on exosomes from other cell types (Fig. 10a) (Escola et al. 1998; Raposo and Stoorvogel 2013). The larger microvesicles (100 nm–1 μm) derive directly from the PM by mechanisms that require platelet activation and calcium-dependent uncoupling of the lipid bilayer from components of the membrane skeleton (Wiedmer et al. 1990; Pasquet et al. 1996). Platelet microvesicles or PMPs are also generated as a result of the shearing forces generated by the flowing blood (Reininger et al. 2006; Cranmer et al. 2011; Tersteeg et al. 2014). Microvesicles and exosomes have been implicated in cell-to-cell communication and represent an important vehicle for interaction with leukocytes (Denzer et al. 2000; Tersteeg et al. 2014). Platelet-derived microvesicles generally have the same surface glycoprotein composition as the activated platelet plasma membrane (i.e., GPIb, the integrins $\alpha_{IIb}\beta_3$ and β1, P-selectin, and CD63). Platelet microvesicles have a high affinity for annexin-V and bind coagulation factors prothrombin and factor X in the presence of calcium. In contrast, platelet exosomes are enriched in cholesterol (Heijnen et al. 2003) and interact poorly with annexin-V, prothrombin, and factor X (Heijnen et al. 1999). Microvesicles and exosomes can function in the direct environment of adhering platelets, at sites of vascular injury, but can also generate long distance heterotypic signaling. Indeed, exosomes are found at contact sites between platelets and neutrophils (Fig. 10b), and PMPs have been observed in several diseases with an inflammatory component, e.g., in the synovial fluid of arthritic joints (Boilard et al. 2010). Platelets interact with leukocytes and monocytes ex vivo (Tersteeg et al. 2014), as well as in vivo in many inflammatory conditions (Weyrich and Zimmerman 2004; Franks et al. 2010). Recent studies have shown that platelet activation induces the synthesis and release of IL-1β (Lindemann et al. 2001), which is synthesized on polyribosomes in the cytoplasm, and can exit the cell via release of vesicles or exosomes. Indeed, IL-1β associated with PMPs supports leukocyte recruitment and the progression of arthritis (Boilard et al. 2010).

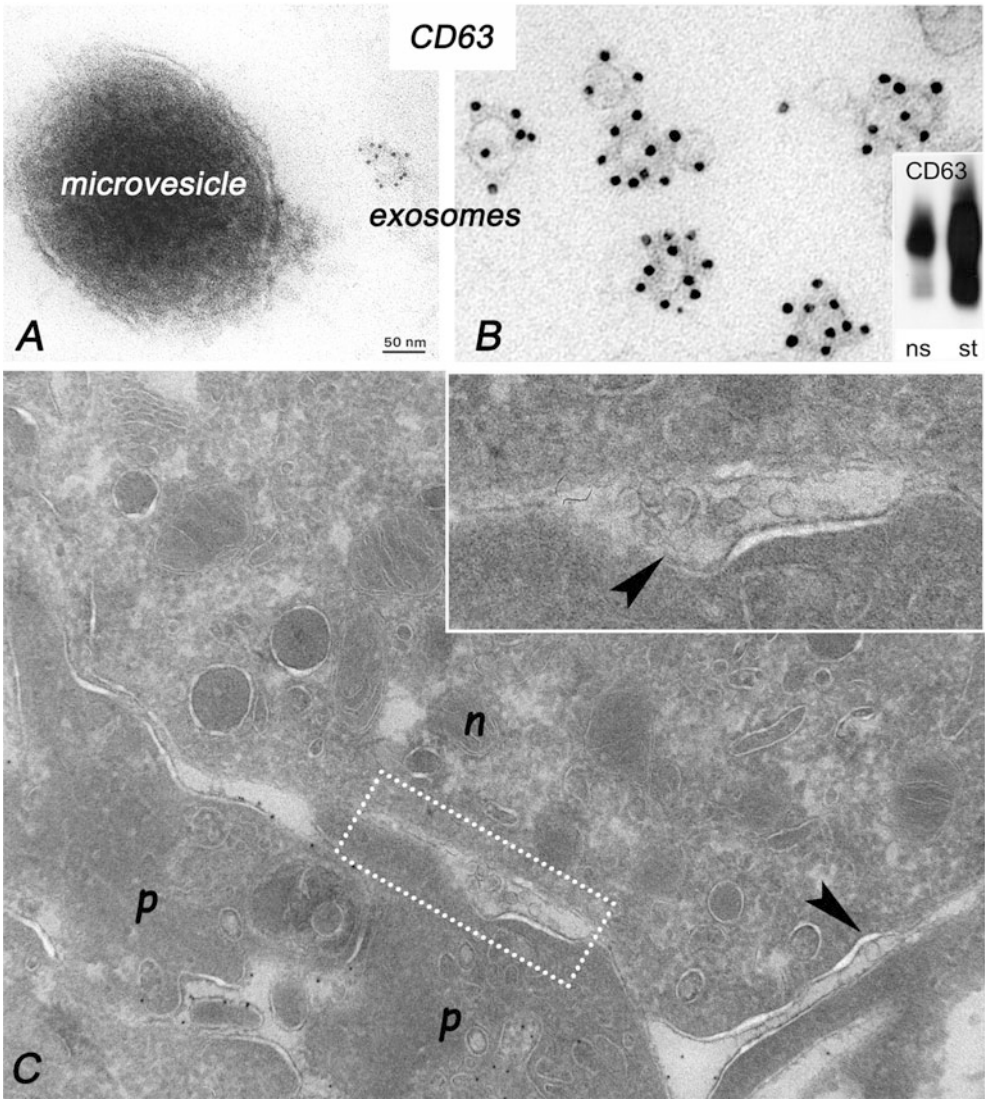

Fig. 10 Microparticles and exosomes. (**a**) Microparticles and exosomes isolated from the secretome of activated platelets. The microparticles contain predominantly cell surface glycoproteins (e.g., GPIb), whereas exosomes are enriched in CD63. *Inset* shows increase of exosome-bound CD63 in the releasate of *st* stimulated platelets. *ns* non-stimulated. (**b**) Neutrophil (N) interacting with adherent platelets (P; GPIb positive). Synaptic area between neutrophil and platelets contains released exosomes (*arrowheads* and highlighted *inset*)

Future Perspectives

The rapid developments in the field of live imaging, the use of sensitive high-speed CCD cameras, increasing subsets of specific fluorophores, and improved image analysis software will increase the applications of "real-time" imaging of platelet adhesion and thrombus formation under flow. It is expected that such methods become also available for high-throughput diagnostic studies of functional adhesion defects and platelet disorders. Rapid developments are also emerging in the field of high-resolution 3D EM, particularly cryo-electron tomography. These techniques open up the way to study (macro)-molecular complexes in their close-to-native cellular context. Such technologies will be increasingly introduced in the platelet field as well and will shed new light on fundamental aspects of platelet structure and function. Ultimately, these advances will lead to their application as high-throughput method, raising the possibility of using it as diagnostic tools in the clinical setting (Wang et al. 2015).

Take-Home Messages

New imaging technologies continuously improve our view on platelet structure and function:

- Live cell imaging is an essential tool to study platelet adhesion and aggregation defects under physiological flow.
- (Immuno)-electron microscopy remains an important tool to study macromolecular features at the ultrastructural level.
- New 3D imaging technologies provide increased insight into the platelet ultrastructure.

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Megakaryocyte Development and Platelet Production

Joseph E. Italiano Jr.

Abstract

Blood platelets are 2–3 μm anucleate fragments that are formed from the megakaryocyte cytoplasm and have a distinctive discoid shape. To generate and release platelets, megakaryocytes undergo endomitosis to become polyploid and follow a maturation program that results in the transformation of the bulk of their cytoplasm into multiple long processes called *proplatelets*. To generate 1000–2000 platelets, a megakaryocyte may extend multiple proplatelets, each of which begins as a thick pseudopodium that over time elongates and branches repetitively. Platelets form predominantly at the tips of proplatelets. As platelets mature, their content of organelles and granules is delivered to them in a flow of individual cargo moving from the cell body of the megakaryocyte to the assembling platelets at the proplatelet ends. Platelet generation can be indiscriminately divided into two stages. The first stage takes days to complete and requires megakaryocyte-specific cytokines, such as thrombopoietin. Substantial nuclear proliferation to 16–32 × N and expansion of the megakaryocyte cytoplasm occur as the platelet is packed with platelet-specific granules, cytoskeletal proteins, and abundant membrane to complete the platelet assembly phase. The second stage is relatively fast and can be completed in hours. During this phase, megakaryocytes generate platelets by reorganizing their cytoplasm first into proplatelets, then preplatelets, which undergo fission to generate platelets. Each day, 100 billion platelets must be generated from megakaryocytes to sustain the normal platelet count of 2–3 × 10^8/mL.

Megakaryocyte Development

Megakaryocytes are large, specialized precursor cells that function to release platelets into the blood circulation. Uncovering mechanisms by which megakaryocytes develop and produce platelets has intrigued scientists for well over a century. Megakaryocytes are derived from pluripotent stem cells and undergo endomitosis, a process in which the cell undergoes multiple DNA replications without cell divisions. During endomitosis, polyploid megakaryocytes initiate a rapid cytoplasmic expansion stage typified by the formation of a highly invaginated demarcation membrane system (DMS) and the amassing of cytoplasmic proteins and granules fundamental for platelet function. During the final stages of development, the megakaryocyte cytoplasm undergoes a spectacular and massive reorganization into long, beaded cytoplasmic extensions called proplatelets. The proplatelets function as the assembly lines of platelet production and ultimately yield individual platelets.

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Commitment to the Megakaryocyte Lineage

Megakaryocytes, like every terminally differentiated hematopoietic cell, originate from hematopoietic stem cells, which are responsible for continuous generation of all circulating blood cells. Hematopoietic cells are categorized by their surface markers, ability to reconstitute host animals, and colony assays that reflect their developmental potential. Hematopoietic stem cells are rare, making up less than 0.1 % of cells in the bone marrow. The development of megakaryocytes from hematopoietic stem cells involves an order of differentiation steps in which the developmental capacities of the progenitor cells become slowly more limited. Hematopoietic stem cells in mice are characteristically identified by the surface markers Lin-Sca-1+c-kit ^{high} (Li and Johnson 1995; Weissman et al. 2001). A comprehensive model of hematopoiesis has been developed from experiments analyzing the effects of hematopoietic growth factors on marrow cells contained in a semisolid media. Hematopoietic stem cells give rise to two major lineages, a common lymphoid progenitor that can develop into lymphocytes and a myeloid progenitor that can develop into eosinophil, macrophage, myeloid, erythroid, and megakaryocyte lineages. A common erythroid-megakaryocytic progenitor arises from the myeloid lineage (Akashi et al. 2000). However, new experiments also suggest that hematopoietic stem cells may directly develop into erythroid-megakaryocyte progenitors (Adolfsson et al. 2005). All hematopoietic progenitors express surface CD34 and CD41, and the commitment to the megakaryocyte lineage is indicated by expression of the integrin CD61 and elevated CD41 levels. From the committed myeloid progenitor cell (CFU-GEMM), there is convincing evidence for a bipotential progenitor intermediate between the pluripotential stem cell and the committed precursor that can develop into biclonal colonies composed of megakaryocytic and erythroid cells. The regulatory pathways and transcriptional factors that allow the erythroid and megakaryocyte lineages to separate from the bipotential progenitor are currently unknown. Diploid precursors that are committed to the megakaryocyte lineage have characteristically been divided into two colonies based on their functional capacities. The megakaryocyte burst-forming cell is a primitive progenitor that has a high proliferation capacity that gives rise to large megakaryocyte colonies. Under specific culture conditions, the megakaryocyte burst-forming cell can develop into 40–500 megakaryocytes within 1 week. The colony-forming cell is a more mature megakaryocyte progenitor that gives rise to a colony containing from 3 to 50 mature megakaryocytes that vary in their proliferation potential. Megakaryocyte progenitors can be readily identified in the bone marrow by immunoperoxidase and acetylcholinesterase labeling. Although both human megakaryocyte colony-forming and burst-forming cells express the CD34 antigen, only colony-forming cells express the HLA-DR antigen.

Several classification systems based on morphological features, biochemical markers, and histochemical staining have been used to categorize different stages of megakaryocyte development. In general, three types of morphologies can be identified in the bone marrow. The promegakaryoblast is the first recognizable megakaryocyte precursor. The megakaryoblast, or stage I megakaryocyte, is a more mature cell that has a well-defined morphology (Long et al. 1982). The megakaryoblast has a kidney-shaped nucleus with two sets of chromosomes (4N). It is 10–50 μm in diameter and looks intensely basophilic in Romanovsky-stained marrow preparations given the large numbers of ribosomes, although the cytoplasm at this stage lacks alpha and dense granules. The megakaryoblast displays a high nuclear-to-cytoplasmic ratio and, in rodents, is acetylcholinesterase positive. The promegakaryocyte, or stage II megakaryocyte, is 20–80 μm in diameter with a polychromatic cytoplasm. The cytoplasm of the promegakaryocyte is less basophilic than the megakaryoblast and now contains developing granules.

Polyploidization

Megakaryocytes, unlike most other cells, become polyploid by undergoing endomitosis, a process which involves repeated cycles of DNA replication without cell division (Ebbe 1976; Ebbe and Stohlman 1965; Odell et al. 1968, 1970; Therman et al. 1983). At the end of the proliferation phase, mononuclear megakaryocyte precursors exit the diploid state to differentiate and undergo endomitosis, resulting in a cell that contains multiples of a normal diploid chromosome content (i.e., 4N, 16N, 32N). Although the number of endomitotic cycles can range from 2 to 6, the majority of megakaryocytes undergo three endomitotic cycles to achieve a DNA content of 16N. Nonetheless, some megakaryocytes can acquire a DNA content as high as 256N. Megakaryocyte polyploidization results in a functional gene amplification whose probable role is an increase in protein synthesis parallel with cell expansion (Raslova et al. 2003). The mechanisms that power endomitosis are not completely understood. It was initially postulated that polyploidization may result from an absence of mitosis after each round of DNA replication. However, recent studies of primary megakaryocytes in culture indicate endomitosis does not result from a complete absence of mitosis, but rather a prematurely terminated mitosis (Raslova et al. 2003; Nagata et al. 1997; Vitrat et al. 1998). Megakaryocyte progenitors initiate the cycle and undergo a short G1

phase, a typical 6–7 h S phase for DNA synthesis, and a short G2 phase followed by endomitosis. Megakaryocytes begin the mitotic cycle and proceed from prophase to anaphase A, but do not enter anaphase B or telophase or undergo cytokinesis. During megakaryocyte polyploidization, the nuclear envelope breaks down and an abnormal spherical mitotic spindle forms. Each spindle attaches chromosomes that align to a position equidistant from the spindle poles (metaphase). Sister chromatids separate and begin to move toward their respective poles (anaphase A). However, the spindle poles fail to move apart and do not undergo the segregation typically observed during anaphase B. Individual chromatids are not moved to the poles and then a nuclear envelope reforms around the complete set of sister chromatids, assembling a single enlarged but lobed nucleus with multiple chromosome copies. The cell then skips telophase and cytokinesis to enter G1. This failure to completely segregate sets of daughter chromosomes may prevent the formation of a nuclear envelope around each individual set of chromosomes. In most cell types, feedback controls and checkpoints ensure that DNA replication and cell division are synchronized. Megakaryocytes appear to be the exception to this rule as they have managed to deregulate this process. Recent work has focused on defining the signals that regulate polyploidization in megakaryocytes (Ravid et al. 2002). It has been suggested that endomitosis may be the consequence of a reduction in mitosis-promoting factor (MPF) activity, a multiprotein complex consisting of Cdc2 and cyclin B (Gu et al. 1999; Wang et al. 1995). MPF contains a kinase activity that is necessary for entry of cells into mitosis. In most cell types, newly generated cyclin B binds to Cdc2 and produces active MPF, while cyclin degradation at the end of mitosis inactivates MPF. Conditional mutations in strains of budding and fission yeast that inhibit either cyclin B or cdc2 cause them to go through an additional round of DNA replication without mitosis (Broek et al. 1991; Hayles et al. 1994). In addition, studies using a human erythroleukemia cell line have established that these cells contain inactive cdc2 during polyploidization, and investigations with phorbol ester-induced Meg T cells have demonstrated that cyclin B is absent in this cell line during endomitosis (Brieher et al. 2006; Datta et al. 1996; Zhang et al. 1996). However, it has been hard to establish the role of MPF activity in promoting endomitosis because these cell lines have a limited ability to undergo endomitosis. Additionally, experiments using normal megakaryocytes in culture have demonstrated normal levels of cyclin B and cdc2 with functional mitotic kinase activity in megakaryocytes undergoing mitosis, suggesting that endomitosis can be regulated by signaling pathways other than MPF. Cyclins appear to play a critical role in directing endomitosis, though a triple knockout of cyclins D1, D2, and D3 does not appear to affect megakaryocyte development (Kozar 2004). Yet, cyclin E-deficient mice do exhibit a profound defect in megakaryocyte development (Geng 2003). It has recently been demonstrated that the molecular programming involved in endomitosis is characterized by the mislocalization or absence of at least two critical regulators of mitosis, the chromosomal passenger proteins Aurora-B/AIM-1 and surviving (Zhang et al. 2004). Whereas deletion of the APC/C cofactor Cdc20 causes mitotic arrest and severe thrombocytopenia, lack of the kinases Aurora-B, Cdk1, or Cdk2 does not affect endomitosis of megakaryocytes or platelet levels. Deletion of Cdk1 forces a change to endocycles without mitosis, whereas polyploidization in the absence of cdk1 and cdk2 occurs in the presence of aberrant replication events. Markedly, ablation of these kinases rescues defects in Cdc20 null megakaryocytes. The observations suggest that endomitosis can be functionally replaced by alternative polyploidization mechanisms in vivo (Trakala et al. 2015).

Cytoplasmic Maturation

During endomitosis, the megakaryocyte begins a maturation stage in which the cytoplasm rapidly fills with organelles, platelet-specific proteins, and membrane systems that will ultimately be organized and packaged into platelets. During maturation, the megakaryocyte enlarges dramatically and the cytoplasm acquires its unique ultrastructural features, including the formation of a demarcation membrane system (DMS), the development of a dense tubular system, and the assembly of granules (Fig. 1). During this phase of megakaryocyte development, the cytoplasm contains loads of ribosomes and rough endoplasmic reticulum, where protein synthesis occurs. One of the most striking features of a mature megakaryocyte is its elaborate DMS, an elaborate network of membrane channels composed of flattened cisternae and tubules. The organization of the cytoplasm into membrane-defined platelet territories was first proposed by Kautz and DeMarsh (1955), and a high-resolution description of this membrane system by Yamada soon followed (Yamada 1957). The DMS is noticeable in early promegakaryocytes, but becomes most prominent in mature megakaryocytes where it permeates the cytoplasm of the megakaryocyte, except for a thin rim of cytoplasm at the cortex from which it is excluded. It has been proposed that the DMS derives from the megakaryocyte plasma membrane in the form of tubular invaginations (Behnke 1968). The DMS is in contact with the external environment and can be labeled with extracellular tracers, such as lanthanum salts, ruthenium red, as well as tannic acid (Behnke 1968; Nakao and Angrist 1968; Zucker-Franklin et al. 1985). The DMS is enriched in polyphosphatidyl 4,5 bisphosphate and the vWf receptor. The exact function of this smooth membrane

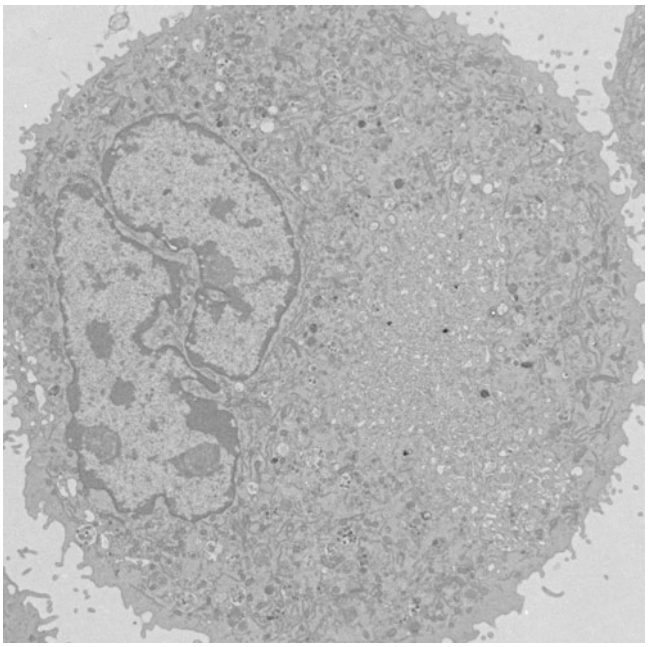

Fig. 1 Transmission electron micrograph of a mature murine megakaryocyte. Overview of one megakaryocyte showing multi-lobulated nucleus, mature cytoplasm, and invaginated membrane system. Megakaryocyte cultures generated from murine fetal liver cells were fixed with 1.25 % paraformaldehyde, 0.03 % picric acid, and 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h, postfixed with 1 % osmium tetroxide, dehydrated through a series of alcohols, infiltrated with propylene oxide, and embedded in epoxy resin. Ultrathin sections were stained and examined with an electron microscope (G2 Spirit BioTwin; Tecnai) at an accelerating voltage of 80 kV. Images were recorded with a CCD camera (2K; Advanced Microscopy Techniques) using digital acquisition and analysis software

system has been debated for many years. Initially, it was proposed to play a key role in platelet production by establishing preformed "platelet territories" within the megakaryocyte cytoplasm (see below). However, more recent experiments suggest that the DMS functions mainly to dramatically increase the apparent membrane to surface ratio, functioning as a membrane reserve for proplatelet formation and extension. Experiments by Eckly et al. have begun to provide understandings into how the DMS forms and matures (Eckly et al. 2014). To develop the DMS, the megakaryocyte plasma membrane enfolds at specific sites and a perinuclear pre-DMS is generated. Next, the pre-DMS is expanded into its mature form by material added from golgi-derived vesicles and endoplasmic reticulum-mediated lipid transfer. This structural description is in line with studies on platelet glycosyltransferases, which arrive early in the developing DMS and eventually make their way to the megakaryocyte and platelet surfaces (Wandall et al. 2012). Only a few proteins have been identified this far to function in DMS formation based on alteration in its structure in certain knockout mouse models. Membrane-deforming proteins that use F-BAR domains to curve membranes, or GTP as an energy source to bud vesicles from membranes, appear to be necessary for normal megakaryocyte maturation and platelet release. Gross disruptions in DMS structure are found in megakaryocytes isolated from either filamin A knockout, dynamin 2 knockout, or Cdc42-interacting protein 4 (CIP4) knockout mice. CIP4 is an F-BAR protein that induces membrane tubulation and localizes to membrane lipids via its BAR domain and interacts with the Wiskott-Aldrich syndrome protein (Chen et al. 2013). CIP4-/- mice have a mild thrombocytopenia with a 25 % decrease in platelet counts. While megakaryocyte numbers and ploidy are normal in CIP4-/- knockout mice, megakaryocytes isolated from these mice are less effective in generating proplatelets in vitro. Dynamins are highly conserved mechanochemical GTPases that function in endocytosis and vesicle transport, and mutations in dynamin 2 have been associated with low platelet count in humans. Dynamin 2-dependent endocytosis is required for development of megakaryocytes in mice (Bender et al. 2015a). The DMS has also been proposed to mature into the open canalicular system (OCS) of the platelet, which functions as a channel for granule secretion. However, bovine megakaryocytes, which have a well-defined DMS, generate platelets that do not form an OCS, suggesting the OCS is not necessarily a remnant of the DMS (Zucker-Franklin et al. 1985).

Platelet Formation

The mechanisms by which blood platelets are produced have been studied for over a century. In 1906, James Homer Wright began a thorough analysis of how giant precursor megakaryocytes give birth to platelets (Wright 1906). Many theories have been proposed to explain how megakaryocytes yield platelets. The DMS, described in detail by Yamada in 1957, was first proposed to demarcate preformed "platelet territories" within the cytoplasm of the megakaryocyte (Yamada 1957). Microscopists recognized that developing megakaryocytes became packed with membranes and platelet-specific organelles and postulated that these membranes formed a system that defined fields for developing platelets (Shaklai et al. 1978). Release of individual platelets was proposed to occur by an immense fragmentation of the megakaryocyte cytoplasm along DMS fracture lines located between these fields. The DMS model proposes that platelets form through an elaborate internal membrane reorganization process (Kosaki 2005). Tubular membranes, which may originate from invagination of the megakaryocyte plasma membrane, are predicted to interconnect and branch, forming a continuous network throughout. The fusion of adjacent tubules has been suggested as a mechanism to generate a flat membrane that ultimately surrounds

the cytoplasm of an assembling platelet. Models attempting to use the DMS to explain how the cell cytoplasm becomes subdivided into platelet volumes and surrounded by its own membrane have lost support because of several inconsistent observations. For example, if platelets are demarcated within the cytoplasm of the megakaryocyte by the DMS, then platelet fields should exhibit the hallmark structural characteristics of resting platelets, which is not the case (Radley and Hatshorm 1987). Platelet territories within the megakaryocyte cytoplasm do not contain microtubule coils, one of the most characteristic features of resting platelet structure. In addition, there are no in vivo studies on living megakaryocytes directly demonstrating that platelet fields explosively fragment or shatter into mature, functional platelets. In contrast, studies that focused on the DMS of megakaryocytes before and after proplatelet retraction induced by microtubule-depolymerizing agents suggest this specialized membrane system may function primarily as a membrane reservoir that evaginates to deliver plasma membrane for the extensive growth of proplatelets (Radley and Haller 1982). Radley and Haller have proposed that the name DMS may be a misnomer and have suggested "invagination membrane system" as a more appropriate name to describe this membrane network.

The Proplatelet Theory

The majority of evidence that has been collected supports the proplatelet model of platelet generation. The term "proplatelet" is generally used to describe long (up to millimeters in length), thin cytoplasmic extensions radiating from megakaryocytes (Fig. 2) (Becker and De Bruyn 1976). These extensions are characterized by multiple platelet-sized beads connected together by thin cytoplasmic bridges and are thought to represent intermediate structures in the megakaryocyte to platelet transition (Fig. 3). The actual concept of platelets arising from these pseudopodia-like structures occurred when Wright recognized that platelets originate from megakaryocytes and described "the detachment of plate-like fragments or segments from pseudopods" emanating from megakaryocytes (Wright 1906). Thiery and Bessis (1956) and Behnke (1969) later described the morphology of these cytoplasmic processes extending from megakaryocytes during platelet formation in more detail. The classic "proplatelet theory" was introduced by Becker and De Bruyn, who proposed that megakaryocytes form long pseudopod-like processes that later fragment to give rise to individual platelets (Becker and De Bruyn 1976). In this early model, the DMS was still proposed to subdivide the megakaryocyte cytoplasm into platelet areas. Radley and Haller later developed the "flow model" which proposed that platelets originate exclusively from the interconnected platelet-sized beads connected along the shaft of proplatelets and suggested that the DMS did not function to define platelet fields but as a reservoir of surface membrane to be evaginated during proplatelet production (Radley and Haller 1982). Developing platelets was assumed to become encased by plasma membrane only as proplatelets were formed.

The bulk of experimental evidence now supports a modified proplatelet model of platelet formation. Proplatelets have been observed:

1. In a wide range of mammalian species, including mice, rat, guinea pigs, dogs, cows, and humans (Choi et al. 1995; Handagama et al. 1987; Leven 1987; Tablin et al. 1990).
2. Both in vivo and in vitro, and maturation of proplatelets yields platelets that are structurally and functionally similar to blood platelets (Choi et al. 1995; Cramer et al. 1997).

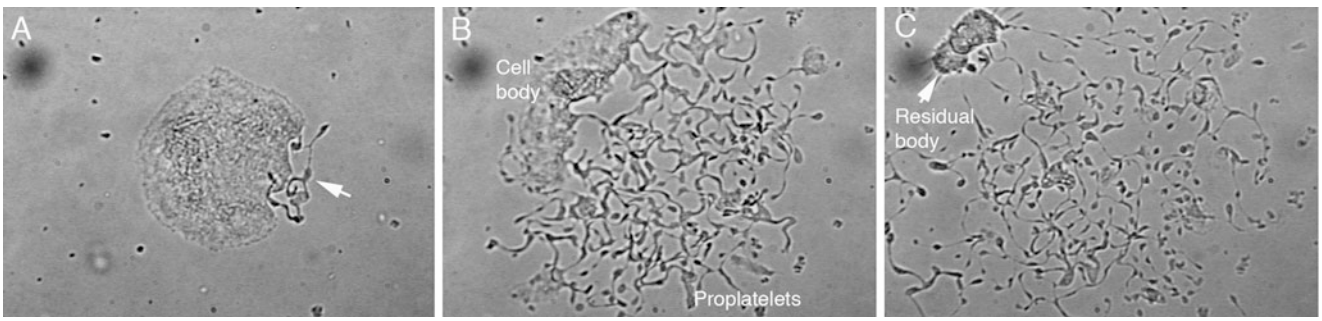

Fig. 2 Formation of proplatelets by a mouse megakaryocyte. Time-lapse sequence of a maturing megakaryocyte, showing the events that lead to elaboration of proplatelets in vitro. (**a**) Platelet production commences when the megakaryocyte cytoplasm starts to erode at one pole. (**b**) The bulk of the megakaryocyte cytoplasm has been converted into multiple proplatelet processes that continue to lengthen and form swellings along their length. These processes are highly dynamic and undergo bending and branching. (**c**) Once the bulk of the megakaryocyte cytoplasm has been converted into proplatelets, the entire process ends in a rapid retraction that separates the released proplatelets from the residual cell body

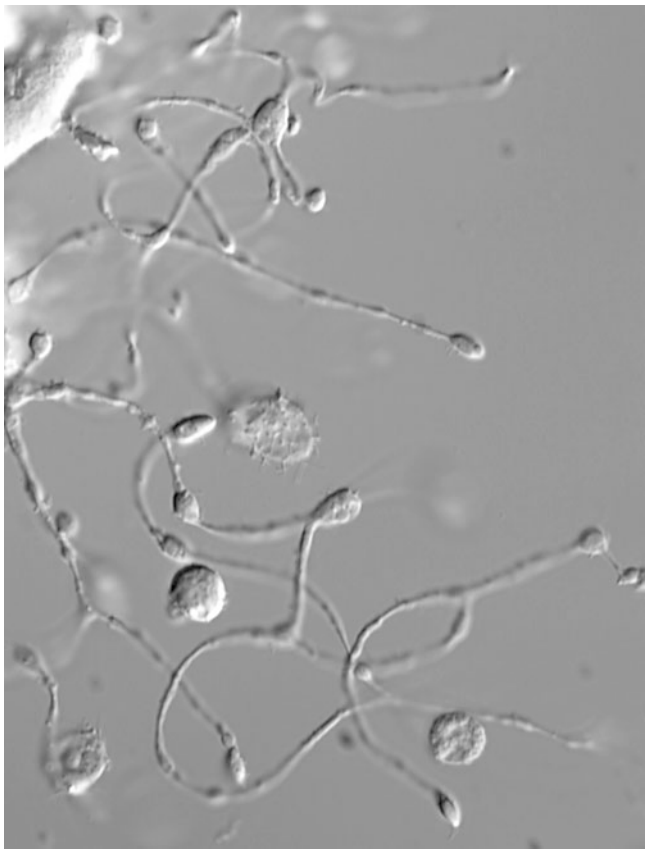

Fig. 3 Structure of proplatelets. Differential interference contrast (DIC) image of proplatelets elaborated by mouse megakaryocytes in culture. Proplatelets contain platelet-sized swellings that decorate their length giving them a beads-on-a-string appearance

3. Extending from megakaryocytes in the bone marrow through junctions in the endothelial lining where they have been hypothesized to be released into circulation and undergo further development into individual platelets (Lichtman et al. 1978; Scurfield and Radley 1981; Tavassoli and Aoki 1989).
4. To be absent in mice lacking two distinct hematopoietic transcription factors. These mice fail to generate proplatelets in vitro and display severe thrombocytopenia (Lecine et al. 1998; Shivdasani et al. 1995, 1997).

Taken together, these findings support an important role for proplatelet formation in thrombopoiesis.

Morphogenesis of Proplatelets

The discovery of thrombopoietin, the major physiological regulator of platelet production, and the development of megakaryocyte cultures that reconstitute platelet formation in vitro has provided systems to study megakaryocytes in the act of forming proplatelets. Time-lapse video microscopy of living megakaryocytes reveals both temporal and spatial changes that lead to the formation of proplatelets (Fig. 2) (Italiano et al. 1999). Conversion of the megakaryocyte cytoplasm concentrates almost all of the intracellular contents into proplatelet processes, and their platelet-sized particles, which in the final stages, appear as beads linked by thin cytoplasmic strings. The transformation unfolds over 5–10 h and commences with the erosion of one pole (Fig. 2) of the megakaryocyte cytoplasm. Thick pseudopodia initially form and then elongate into thin tubes of uniform diameter of 2–4 μm. These slender tubules, in turn, undergo a dynamic bending and branching process and form periodic densities along their length. Ultimately, the megakaryocyte is transformed into a "naked" nucleus surrounded by an extensive network of proplatelet processes. Megakaryocyte maturation ends when a rapid retraction separates the proplatelets from the cell body releasing them into culture (Fig. 2). The subsequent rupture of the cytoplasmic bridges between platelet-sized segments is believed to release individual platelets.

The Cytoskeleton Powers Platelet Production

The cytoskeleton of the mature platelet plays a crucial role in maintaining the discoid shape of the resting platelet and is responsible for the shape change that occurs during platelet activation. This same set of cytoskeletal proteins provides the force to bring about the shape changes associated with megakaryocyte maturation (Patel et al. 2005). Three cytoskeletal polymer systems exist in megakaryocytes: actin, tubulin, and spectrin. These proteins reversibly assemble into cytoskeletal filaments. Evidence supports a model of platelet production in which microtubules, actin filaments, and the spectrin-based membrane skeleton play key roles.

Microtubules Drive Proplatelet Extension Microtubules were first extensively studied in resting platelets. One of the most distinguishing features of the resting platelet is its marginal microtubule coil (White and Krivit 1967). αβ-Tubulin dimers assemble into microtubules under physiologic conditions, and in resting platelets, tubulin is equally divided between dimer and polymer fractions. In many cell types, the αβ-tubulin subunits are in a dynamic equilibrium with microtubules such that reversible cycles of assembly-disassembly of microtubules are observed. Microtubules are long, hollow polymers, 24 nm in diameter, and responsible for many types of cellular movements, such as the transport of organelles across the cell and the segregation of chromosomes during mitosis. The microtubule coil of the resting platelet, initially characterized in the late 1960s by Jim White, has been described as a single microtubule

approximately 100 μm long and is coiled 8–12 times inside the periphery of the platelet (White and Krivit 1967). The primary function of the microtubule coil is to maintain the disk shape of the resting platelet. Depolymerization of platelet microtubules with drugs such as vincristine, colchicine, or nocodazole causes platelets to become spherical and lose their discoid shape (White and Krivit 1967). Chilling platelets to 4 °C also causes disassembly of the microtubule coil and loss of the discoid shape. Furthermore, elegant studies show that mice lacking the major hematopoietic β-tubulin isoform (β1 tubulin) contain platelets that lack the characteristic disk shape and have defective marginal bands. Genetic elimination of β1 tubulin in mice results in low platelet count with mice containing circulating platelet counts below 50 % of normal. β1 tubulin-deficient platelets are spherical in shape and this appears to be due to defective marginal bands with fewer microtubule coilings. Whereas normal platelets possess a marginal band that consists of 8–12 coils, β1 tubulin knockout platelets contain only 2–3 coils (Schwer et al. 2001). Human β1 tubulin functional substitution (AG > CC) inducing both structural and functional platelet alterations has been described (Freson et al. 2005). The Q43P β1-tubulin variant was found in 10.6 % of the general population and in 24.2 % of 33 unrelated patients with undefined congenital macrothrombocytopenia. Electron microscopy revealed enlarged spherocytic platelets with a disrupted marginal band and structural alterations. Platelets with the Q43P β1-tubulin variant showed a mild platelet dysfunction, with reduced adenosine triphosphate (ATP) secretion, thrombin-receptor-activating peptide (Browne et al. 2000)-induced aggregation, and impaired adhesion to collagen under flow conditions. A more than doubled prevalence of the β1-tubulin variant was observed in healthy subjects not undergoing ischemic events, suggesting it could confer an evolutionary advantage and protective cardiovascular role. The microtubules that make up the coil are coated with proteins that regulate polymer stability (Kenney and Linck 1985). The microtubule motor proteins kinesin and dynein have been localized to platelets, but their roles in resting and activated platelets have not yet been defined.

Proplatelet formation is contingent on microtubule function as exposure of megakaryocytes to drugs that depolymerize microtubules, such as vincristine or nocodazole, blocks proplatelet production. Microtubules, hollow polymers assembled from αβ-tubulin dimers, are the major structural components of the engine that powers proplatelet elongation. Examination of the microtubule cytoskeletons of proplatelet-producing megakaryocytes provides insights as to how microtubules power platelet production (Fig. 4) (Patel, 2005 #4018). The microtubule cytoskeleton in megakaryocytes undergoes a spectacular reorganization during proplatelet production. In immature megakaryocytes without proplatelets, microtubules radiate out from the cell center (centrosome) to the cortex. As thick pseudopods form during the initiation of proplatelet formation, membrane-associated microtubules organize into thick bundles positioned just beneath the plasma membrane of these structures. And once pseudopodia begin to extend (at an average rate of 1 μm/min), microtubules form linear arrays that line the whole length of the proplatelet processes (Fig. 4). The microtubule bundles are thickest in the proplatelet near the body of the megakaryocyte but thin to bundles of approximately seven microtubules near proplatelet tips (Fig. 5). The distal end of each proplatelet always has a platelet-sized enlargement that contains a microtubule bundle that loops

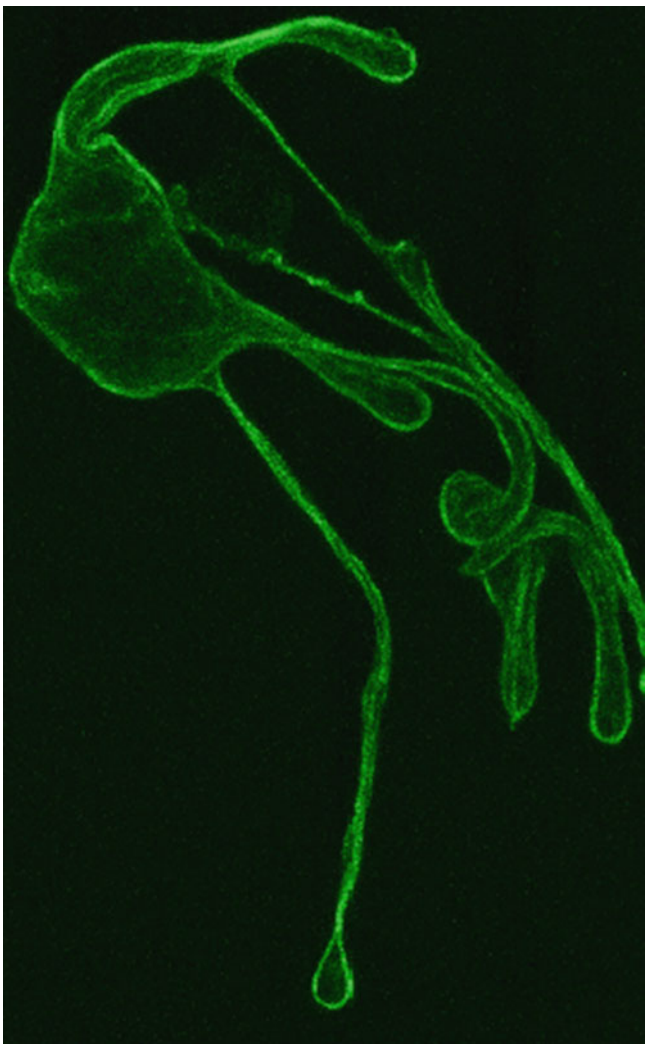

Fig. 4 Localization of microtubules within proplatelets. Immunofluorescence studies on murine megakaryocytes grown in culture and labeled with β1-tubulin antibodies indicate that microtubules line the entire length of proplatelets. The hallmark features of proplatelets, including the tip, swellings, shafts, and branch points, are visible

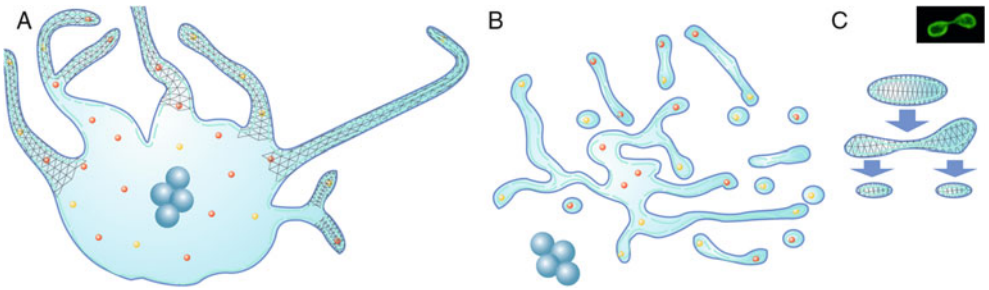

Fig. 5 Model of platelet production. (**a**) The formation of proplatelets begins with the extension of thick pseudopodia that use cortical bundles of microtubules to extend and form thin proplatelets with bulbous ends. Proplatelet membranes are laminated with an undercoat of spectrin. The ends of proplatelets contain a bundle of microtubules that loop on themselves. Proplatelet elongation involves the sliding of microtubules past one another, driven by the molecular motor cytoplasmic dynein. As proplatelets extend, development of the membrane surface area necessitates the outflow of the invaginated membrane reservoir, a process that requires reorganization of the membrane skeleton. Mitochondria and granules traffic (as indicated by the *orange* and *yellow spheres*) to the tips of proplatelets along microtubules, which function as the highways of the cell. Actin promotes the branching and amplification of proplatelet tips, representing a mechanism to augment the numbers of proplatelet tips and ultimately, platelets. (**b**) The entire megakaryocyte cytoplasm is converted into a mass of proplatelets. (**c**) Proplatelets continue to morph into preplatelets (anucleate discoid particles 2–10 μm across), which are released from the cell. Preplatelets reversibly convert into barbell proplatelets (*inset*, *top right*), a process that is driven by microtubule-based forces. The membrane skeleton stabilizes this barbell form. Platelets release from proplatelet ends after the final fission event. The nucleus is eventually extruded from the proplatelets, ending the role of the megakaryocyte in this process

just beneath the plasma membrane and reenters the shaft to form a teardrop-shaped or tennis racket-shaped structure. Because microtubule coils similar to those observed in blood platelets are detected only at the ends of proplatelets and not within the platelet-sized beads found along the length of proplatelets, mature platelets are formed predominantly at the ends of proplatelets.

Direct visualization of microtubule dynamics in living megakaryocytes using green fluorescent protein (GFP) technology has provided a window into how microtubules power proplatelet elongation (Patel et al. 2005). End-binding protein three (EB3), a microtubule plus end-binding protein associated only with growing microtubules, fused to GFP was retrovirally expressed in murine megakaryocytes and used as a marker to follow microtubule plus end dynamics. Immature megakaryocytes without proplatelets employ a centrosomal-coupled microtubule nucleation/assembly reaction, which appears as a prominent starburst pattern when visualized with EB3-GFP. Microtubules assemble only from the centrosomes and grow outward into the cell cortex where they turn and run in parallel with the cell edges. However, just before proplatelet production begins, centrosomal assembly stops and microtubules begin to consolidate into the cortex. Fluorescence time-lapse microscopy of living, proplatelet-producing megakaryocytes expressing EB3-GFP reveals that as proplatelets elongate, microtubule assembly occurs continuously throughout the entire proplatelet, including the swellings, shaft, and tip. The rates of microtubule polymerization (average of 10.2 μm/min) are approximately tenfold faster than the proplatelet elongation rate, suggesting polymerization and proplatelet elongation are not tightly coupled. The EB3-GFP studies also revealed that microtubules polymerize in both directions in proplatelets, e.g., both toward the tips and cell body, demonstrating that the microtubules composing the bundles have a mixed polarity.

Even though microtubules are continuously polymerizing in proplatelets, polymerization does not provide the force for proplatelet extension. Proplatelets continue to elongate even when microtubule polymerization is blocked by drugs that inhibit net microtubule assembly, suggesting an alternative mechanism for proplatelet extension (Patel et al. 2005). Consistent with this idea, proplatelets possess an inherent microtubule sliding mechanism. Cytoplasmic dynein, a minus-end microtubule molecular motor protein, localizes along the microtubules of the proplatelet and appears to directly contribute to microtubule sliding, since inhibition of dynein, through disassembly of the dynactin complex, prevents proplatelet formation. Microtubule sliding can also be reactivated in detergent-permeabilized proplatelets. ATP, known to support the enzymatic activity of microtubule-based molecular motors, activates proplatelet elongation in permeabilized proplatelets that contain both cytoplasmic dynein and its regulatory complex, dynactin. More recent analysis has indicated six types of behaviors characterize the elaboration of proplatelets: elongation, branching, pausing, fusions, fragmentations, and retractions. While the average elongation rate for proplatelets over time is 1 μm/min, extension normally occurs in bursts and pauses. Burst rates greatly exceed the average rates and under flow, and rates of >30 μm/min have been observed. These rates correlate well with the sliding rates of microtubules within

the bundles. Fluorescence recovery after photobleaching studies have demonstrated that microtubule sliding drives proplatelet elongation and is dependent on cytoplasmic dynein (Bender et al. 2015b). Thus, dynein-facilitated microtubule sliding appears to be the key event in driving proplatelet elongation.

Transport of Organelles and Granules into Assembling Platelets In addition to playing an essential role in proplatelet elongation, the microtubules lining the proplatelets serve a second function—the transport of membrane, organelles, and granules into proplatelets and assembling platelets at proplatelet ends. Individual organelles are sent from the cell body into the proplatelets where they move back and forth until they are captured at proplatelet tips (Richardson et al. 2005). Electron microscopy and immunofluorescence studies indicate that organelles are intimately associated with microtubules, whereas actin poisons do not block organelle motion. Thus, movement appears to involve microtubule-based forces. Bidirectional organelle movement is conveyed in part by the bipolar organization of microtubules within the proplatelet, as kinesin-coated latex beads move in both directions over the microtubule arrays of permeabilized proplatelets. Of the two major microtubule motors, kinesin and dynein, only the plus-end directed kinesin is localized in a punctate pattern similar to organelles and granules and is likely responsible for transporting these cargo along microtubules (Richardson et al. 2005). It appears that a twofold mechanism of organelle and granule movement occurs in platelet assembly. First, organelles and granules travel along microtubules, and second, the microtubules themselves can slide bidirectionally in relation to other motile filaments to indirectly move organelles along proplatelets in a piggyback manner.

Actin-Driven Branching and Elaboration of Proplatelets Actin, at a concentration of 0.5 mM, is the most plentiful of all the platelet proteins with two million molecules expressed per platelet (Nachmias and Yoshida 1988). Like tubulin, actin is in a very dynamic monomer-polymer equilibrium. Forty percent of the actin subunits polymerize to form the 2000–5000 linear actin filaments present in the resting cell (Hartwig and DeSisto 1991). The rest of the actin in the platelet cytoplasm is maintained in storage as a 1–1 complex with β4-thymosin (Safer and Nachmias 1994) and is converted to filaments during platelet activation to drive cell spreading. All evidence indicates that the filaments of the resting platelet are interconnected at various points into a rigid cytoplasmic network, as platelets express high concentrations of actin-crosslinking proteins including filamin and α-actinin (Rosenberg and Stracher 1982; Rosenberg et al. 1981). Both filamin and α-actinin are homodimers in solution. Filamin subunits are elongated strands composed primarily of 24 repeats, each ~100 amino acids in length that are folded into IgG-like beta barrels (Fucini et al. 1997; Gorlin et al. 1990). There are three filamin genes on chromosomes 3, 7, and X. Filamin A (X) (Gorlin et al. 1993) and filamin B are expressed in platelets with filamin A being present at greater than tenfold excess to filamin B. Filamin is now recognized to be a prototypical scaffolding protein that attracts binding partners and positions them adjacent to the plasma membrane (Stossel et al. 2001). Partners bound by filamin members include the small GTPase, RalA, Rac, Rho, and Cdc42 with RalA binding in a GTP-dependent manner (Ohta et al. 1999), the exchange factors Trio and Toll, kinases such as PAK1, phosphatases, and transmembrane proteins. Essential to the structural organization of the resting platelet is an interaction that occurs between filamin and the cytoplasmic tail of the GPIbα subunit of the GPIb-IX-V complex. The second rod domain (repeats 17–20) of filamin has a binding site for the cytoplasmic tail of GPIbα, and biochemical experiments have shown that the bulk of platelet filamin ($\geq$90 %) is in complex with GPIbα (Kovacsovics et al. 1995). This interaction has three consequences. First, it positions filamin's self-association domain and associated partner proteins at the plasma membrane while presenting filamin's actin-binding sites into the cytoplasm. Second, because a large fraction of filamin is bound to actin, it aligns the GPIb-IX-V complexes into rows on the surface of the platelet over the underlying filaments. Third, because the filamin linkages between actin filaments and the GPIb-IX-V complex pass through the pores of the spectrin lattice, it restrains the molecular movement of the spectrin strands in this lattice and holds the lattice in compression. The FLN-GPIbα connection is essential for the formation and release of discoid platelets by megakaryocytes as platelets lacking this connection are large and fragile and produced in low numbers. However, the role of the filamin-GPIbα connection in platelet construction per se is not fully clear. Because a low number of Bernard-Soulier platelets form and release from megakaryocytes, it can be argued that this connection is a late event in the maturation process and is not per se required for platelet shedding.

Each megakaryocyte has been estimated to release thousands of platelets (Harker 1978; Harker and Finch 1969; Kaufman et al. 1965; Trowbridge et al. 1984). Analysis of time-lapsed video microscopy of proplatelet development from megakaryocytes grown in vitro has revealed that ends of proplatelets are amplified in a dynamic process that repeatedly bends and bifurcates the proplatelet shaft (Italiano et al. 1999). Amplification of proplatelet tips initiates when a proplatelet shaft is bent into a sharp kink, which then folds back on itself, forming a loop in the microtubule bundle. The new loop eventually elongates, forming a new proplatelet shaft branching from the side of the original

proplatelet. Loops lead the proplatelet tip and define the site where nascent platelets will assemble and where platelet-specific contents are trafficked. In marked difference to the microtubule-based motor that elongates proplatelets, actin-based force is used to bend the proplatelet in end amplification. Megakaryocytes treated with the actin toxins, cytochalasin, or latrunculin can only extend long, unbranched proplatelets that are decorated with few swellings along their length. Despite extensive characterization of actin filament dynamics during platelet activation, how actin participates in this reaction and the cytoplasmic signals that regulate bending have yet to be determined. Electron microscopy and phalloidin staining of megakaryocytes undergoing proplatelet formation indicate that actin filaments are distributed throughout the proplatelet and are particularly abundant within swellings and at proplatelet branch points. One possibility is that proplatelet bending and branching is driven by the actin-based molecular motor myosin. A genetic mutation in the nonmuscle myosin heavy chain-A gene MYH9 in humans results in a group of diseases comprising May-Hegglin anomaly and (the) Sebastian, Fechtner, and Epstein syndromes, characterized by thrombocytopenia with giant platelets. Studies also indicate that protein kinase Cα (PKCα) associates with aggregated actin filaments in megakaryocytes undergoing proplatelet formation and inhibition of PKCα or integrin signaling pathways prevent actin filament aggregation and proplatelet formation in megakaryocytes (Rojnuckarin and Kaushansky 2001). Because proplatelets elongate, but do not branch in the presence of the actin-disrupting drug cytochalasin B, it is unexpected that the deletion of certain actin-associated proteins from the megakaryocyte lineage leads to macrothrombocytopenia. It seems likely that the removal of actin-modulating proteins alters and/or increases filamentous actin and the cytoskeletal structure appears to have a dominant inhibitory effect on proplatelet production and release. The absence of the cytoplasmic actin-crosslinking protein filamin A in conditional mice whose megakaryocyte lineage lacks filamin A results in macrothrombocytopenia in which platelet counts are reduced by 80–90 % (Begonja et al. 2011). Conditional mice lacking the actin turnover protein cofilin-1 in the megakaryocyte lineage also contain giant platelets with a platelet count reduced 60–80 % of normal. In contrast, mice lacking the actin turnover protein ADF have normal platelet counts and morphology (Bender et al. 2010). On the other hand, when ADF-/- mice are crossed with cofilin-1 knockout mice, platelet production is severely reduced and morphologies of platelets are highly variable. Mice that contain megakaryocytes that specifically lack profilin 1, a small protein that promotes actin filament assembly, have macrothrombocytopenia with reduced platelet counts (Bender et al. 2014). Profilin-null platelets have a thickened microtubule coil with hyper-acetylated microtubules, and in some ways, the profiling 1 knockout phenotype is similar to the behavior of platelets in Wiskott-Aldrich syndrome, or in WASp knockout mice. Defective proplatelet production has also been observed in mice in which the small regulatory GTPases Rho, Cdc42, and Rac have been deleted in the megakaryocyte lineage (Pleines et al. 2012, 2013).

The Spectrin Membrane Skeleton Whereas the functions of microtubules and actin filaments in proplatelet production have been extensively studied, our understanding of the role of the membrane skeleton has only recently been recognized. The OCS and plasma membrane of the resting platelet are supported by an elaborate cytoskeletal system. The platelet is the only other cell besides the red blood cell whose membrane skeleton has been visualized at high resolution. Like the erythrocyte, the platelet membrane skeleton is also a self-assembly of elongated spectrin strands that interconnect through their binding to actin filaments generating triangular pores. Platelets contain approximately 2000 spectrin molecules (Hartwig and DeSisto 1991; Fox et al. 1987, 1988). This spectrin network coats the cytoplasmic surface of both the OCS and plasma membrane systems. Although considerably less is known about how the spectrin-actin network forms and is connected to the plasma membrane in the platelet relative to the red blood cell, certain differences between the two membrane skeletons have been defined. First, the spectrin strands composing the platelet membrane skeleton interconnect using the ends of long actin filaments instead of short actin oligomers (Hartwig and DeSisto 1991). These ends arrive at the plasma membrane originating from filaments in the cytoplasm. Hence, the spectrin lattice is organized into a continuous network by its association with actin filaments. Second, tropomodulins are not expressed at sufficiently high levels, if at all, to have a major role in the capping of the pointed ends of the platelet actin filaments; instead, biochemical experiments have revealed that a substantial number (~2000) of these ends are free in the resting platelet. Third, although little tropomodulin protein is expressed, α-adducin is abundantly expressed and appears to cap many of the barbed ends of the filaments composing the resting actin cytoskeleton (Barkalow et al. 2003). Adducin is a key component of the membrane skeleton forming a triad complex with spectrin and actin. Capping of barbed filament ends by adducin also serves the function of targeting them to the spectrin-based membrane skeleton, as the affinity of spectrin for adducin-actin complexes is greater than for either actin or adducin alone (Kaiser et al. 1989; Kuhlman et al. 1996; Matsuoka et al. 1998, 2000). High-resolution electron microscopy reveals that proplatelets contain a spectrin-based membrane

skeleton similar in structure to that of blood platelets (Patel-Hett et al. 2011). Spectrin tetramer assembly is essential for the production of the DMS and proplatelet elaboration, as expression of a spectrin tetramer-disrupting peptide in megakaryocytes inhibits the progression of both processes. In addition, incorporation of this spectrin-disrupting construct into a detergent-permeabilized model system quickly destabilizes proplatelet morphology, causing massive swelling and blebbing. Spectrin tetramers also stabilize the barbell-shaped structures that give rise to individual platelets. Overall, these observations suggest a key role for spectrin in distinct steps of megakaryocyte development through its participation in the generation of the DMS and in the preservation of proplatelet structure.

Release of Individual Platelets

In vivo, proplatelets protrude into bone marrow sinusoids, where they are released and enter the bloodstream. Junt and colleagues as well as Massberg and colleagues have used intravital multiphoton microscopy to visualize proplatelet production in the opened cranial marrow cavity of living mice (Junt et al. 2007; Zhang et al. 2012). Fluorescently-labeled megakaryocytes could be observed to protrude proplatelets and release megakaryocyte fragments into the marrow sinusoids of living mice. Remarkably, these anucleate megakaryocyte fragments typically exceed platelet dimensions, suggesting that platelet morphogenesis continues in the circulation. Consistent with these observations, we have recently identified a previously unrecognized intermediate stage in platelet production and release, which we called the preplate (Thon et al. 2010). Preplatelets, which appear as "giant platelets," are classified as discoid cells (3–10 μm) that retain the capacity to convert into barbell-shaped proplatelets and undergo fission into platelets. Inhibitors of microtubule assembly block the conversion of preplatelets to barbells. Thus, the conversion of preplatelets to barbell proplatelets is powered by microtubule-based forces. It is tempting to speculate that the preplatelet fission reaction is a major regulator of platelet size and that some giant platelet disorders may represent a failure to convert preplatelets into barbell proplatelets. Force constraints resulting from cortical microtubule band diameter and thickness appear to regulate barbell-proplatelet formation, and platelet size is likely limited by microtubule bundling, elastic bending, and actin-myosin-spectrin cortex forces (Thon et al. 2012). Recently, it was demonstrated that individual human platelets have the innate capacity to duplicate and form new cell bodies that undergo fission into platelets (Schwertz et al. 2010). The morphological similarities between platelets that form new cell bodies and preplatelets are striking. Whether newly released platelets exhibit a preplatelet phenotype, which may allow them to form barbell shapes and divide again, is unclear.

Regulation of Platelet Formation In Vivo

While megakaryocyte maturation and platelet production have been extensively studied in vitro, studies analyzing the development of megakaryocytes in their in vivo environment have clearly lagged behind. Even though megakaryocytes arise in the bone marrow, they can migrate into the bloodstream and as a consequence platelet formation may also occur at non-marrow sites. Platelet biogenesis has been proposed to take place in many different tissues, including the bone marrow, lungs, and blood. Specific stages of platelet development have been observed in all three locations. Megakaryocytes cultured in vitro outside the borders of the bone marrow can form well-developed proplatelets in suspension, suggesting direct interaction with the marrow environment is not essential for platelet production. Nonetheless, the effectiveness of platelet production in culture appears to be reduced relative to that observed in vivo, and the bone marrow environment composed of a complex adherent cell population could play a role in platelet production by direct cell contact or secretion of cytokines. Scanning electron micrographs of bone marrow megakaryocytes with proplatelets protruding through junctions in the endothelial lining into the sinusoidal lumen have been published, suggesting platelet production occurs in the bone marrow (Behnke 1969; Lichtman et al. 1978; Scurfield and Radley 1981; Radley 1986; Radley and Scurfield 1980). Bone marrow megakaryocytes are strategically positioned in the extravascular space on the abluminal side of sinus endothelial cells and appear to extend proplatelet projections into the lumen of sinusoids. Electron micrographs show that these cells are anchored to the endothelium by organelle-free projections extended by the megakaryocytes. Several observations suggest that thrombopoiesis is dependent on the direct cellular interaction of megakaryocytes with BMECs specific adhesion molecules (Kopp et al. 2005). It has been demonstrated that the translocation of megakaryocyte progenitors to the vicinity of bone marrow vascular sinusoids was sufficient to induce maturation of megakaryocytes (Avecilla et al. 2004). Implicated in this process are the chemokines FGF-4 and SDF-1, which are known to stimulate expression of adhesion molecules, including very late antigen (VLA)-4 on megakaryocytes and VCAM-1 on BMECs (Avraham et al. 1993, 1994). Disruption of BMEC VE-cadherin-mediated homotypic intercellular adhesion interactions results in a profound inability of the vascular niche to support

megakaryocyte differentiation and to act as a conduit to the bloodstream.

Whether individual platelets are released from proplatelets into the sinus lumen or whether megakaryocytes preferentially release large proplatelet processes into the sinus lumen which later fragment into individual platelets within the circulation is not fully clear. Behnke and Forer have suggested that the final stages of platelet development occur solely in the blood circulation (Behnke and Forer 1998). In this model of thrombopoiesis, megakaryocyte fragments released into the blood become transformed into platelets while in circulation. This theory is supported by several observations:

First, the presence of megakaryocytes and megakaryocyte processes that are sometimes beaded in blood has been amply documented. Megakaryocyte fragments can represent up to 5–20 % of the platelet mass in plasma.

Second, these megakaryocyte fragments, when isolated from platelet-rich plasma, have been reported to elongate, undergo curving and bending motions, and eventually fragment to form disk-shaped structures resembling chains of platelets.

Third, since both cultured human and mouse megakaryocytes can form functional platelets in vitro, neither the bone marrow environment nor the pulmonary circulation is essential for platelet formation and release.

Lastly, many of the platelet-sized particles generated in these in vitro systems still remain attached by small cytoplasmic bridges.

It is possible that the shear forces encountered in circulation or an unidentified fragmentation factor in blood may play a crucial role in separating proplatelets into individual platelets. In response to acute platelet needs during periods of stress, thick protrusion release and megakaryocyte fragmentation have been recently described (Kowata et al. 2014; Nishimura et al. 2015).

Megakaryocytes have been visualized in intravascular sites within the lung, leading to the hypothesis that platelets are formed from their parent cell in the pulmonary circulation (Aschoff 1893). Aschoff first described pulmonary megakaryocytes and proposed that they originated in the marrow, migrated into the bloodstream, and, because of their massive size, were lodged in the capillary bed of the lung where they produced platelets. This mechanism requires the movement of megakaryocytes from the bone marrow into the circulation. Although the size of megakaryocytes would seem limiting, the transmigration of entire megakaryocytes through endothelial apertures of approximately 3–6 μm in diameter into the circulation has been observed in electron micrographs and by early living microscopy of rabbit bone marrow (Tavassoli and Aoki 1989). Megakaryocytes express the chemokine receptor CXCR4 and can respond to the CXCR4 ligand stromal cell-derived factor 1 (SDF-1) in chemotaxis assays. However, both mature megakaryocytes and platelets are non-responsive to SDF-1 suggesting the CXCR4 signaling pathway may be turned off during late stages of megakaryocyte development. This may provide a simple mechanism for retaining immature megakaryocytes in the marrow and permitting mature megakaryocytes to enter the circulation where they can liberate platelets. In humans, megakaryocytes are ten times more concentrated in pulmonary arterial blood than in blood obtained from the aorta (Levine and Willard 1983). In spite of these observations, the estimated contribution of pulmonary megakaryocytes to total platelet production remains unclear, as values have been estimated from 7 to 100 %. Experimental results using accelerated models of thrombopoiesis in mice clearly demonstrate that the fraction of platelet production occurring in the murine lung is insignificant. Davis and colleagues reported that megakaryocytes and their naked nuclei were rarely observed in lung tissue even after strong stimulation of thrombopoiesis.

Conclusion

Megakaryocytes are highly specialized precursor cells that function exclusively to generate and release platelets. After completing endomitosis, polyploid megakaryocytes undergo a rapid cytoplasmic expansion phase characterized by the formation of an extensive demarcation membrane system and the accumulation of cytoplasmic proteins and granules critical for platelet function. During the final stages of development, the megakaryocyte cytoplasm undergoes a massive remodeling in cytoplasmic extensions termed proplatelets. Proplatelets function as the assembly lines of platelet production. Microtubules, actin filaments, and the spectrin membrane skeleton all contribute to the formation of proplatelets and production of individual platelets.

Take-Home Messages

- Hundred billion new platelets are produced daily from bone marrow megakaryocytes in order to maintain platelet counts of 150–400 $\times$ 10^9 platelets/L of whole blood.
- Megakaryocytes are derived from hematopoietic stem cells in the bone marrow and migrate from the osteoblastic niche to the vascular niche during maturation.

(continued)

- Megakaryocytes undergo endomitosis and cytoplasmic maturation as they undergo cytoplasmic maturation.
- Mature megakaryocytes remodel their cytoplasm into long cytoplasmic processes called proplatelets; they extend into sinusoidal blood vessels.
- Proplatelets function as the assembly lines of platelet production.
- The cytoskeleton provides the force to power the elaboration of proplatelets.
- Growth hormones and extracellular matrix proteins in the bone marrow direct hematopoietic stem cell differentiation, megakaryocyte maturation, and proplatelet production.
- Platelet release occurs in the vasculature through barbell proplatelet and circular preplatelet intermediates.

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Platelet Heterogeneity

Shawn Jobe

Abstract

The heterogeneity of platelets is present at rest, upon stimulation by agonists, and within the hemostatic plug. Circulating platelets are heterogeneous in size, age, and responsiveness. Differences in platelet size, platelet age, and platelet genetics can result in variable reactivity and altered interactions of platelets both within and between individuals. Following stimulation by strong agonists, distinct activated platelet subpopulations of spread and aggregating platelets and procoagulant platelets can be identified. Procoagulant platelets differ in morphology and function from spread and aggregating platelets. Finally, morphologically and functionally distinct platelet subpopulations participate in the spatiotemporal regulation of hemostatic plug and thrombus formation.

Platelet Size Heterogeneity

Circulating platelets vary in size from less than 2 to >15 μm^3 during steady-state hematopoiesis (Paulus 1975). This wide variation differs from the more homogeneously sized circulating white and red blood cell populations. Increased platelet size has been correlated with increased platelet reactivity and function. Larger platelet size has been correlated with increased aggregability to physiological agonists, increased dense granule number and release of dense granule contents, and increased surface density of the fibrinogen receptor, $\alpha_{IIb}\beta_3$ (Karpatkin 1978; Thompson et al. 1982; Giles et al. 1994). A prevalence of young, reticulated platelets among larger platelets may contribute to this increased reactivity. In patients treated with multiple antiplatelet agents, residual platelet reactivity of platelets to agonist stimulation is associated with higher numbers of circulating immature platelets and a higher mean platelet volume (MPV) (Cesari et al. 2008; Guthikonda et al. 2008).

The inclusion of mean platelet volume as a measured variable in automated cell counters allows its ready evaluation as a potential risk-assessment tool. This has led to numerous studies investigating potential association with inflammatory and thrombotic diseases. Although readily measured, a number of preanalytic conditions can affect the MPV. The use of EDTA-anticoagulated blood and a standard time between collection and measurement is currently recommended to minimize these differences (Lancé et al. 2012, 1993). A significant increase in MPV has been associated with greater likelihood of a plethora of conditions and events, among these being complications of obstructive sleep apnea (Sökücü et al. 2014), erectile dysfunction (Otunctemur et al. 2015), morbidity in patients with infective endocarditis (Gunebakmaz et al. 2010), progression to hepatocellular carcinoma in chronic liver disease (Kurt et al. 2012), and even likelihood of mortality in patients admitted to the intensive care unit (Zampieri et al. 2014). Best studied are thrombotic events, both arterial and venous, and their association with MPV.

Numerous studies have demonstrated an association of MPV with both the initial incidence and recurrence of acute

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myocardial infarction and its complications (Martin et al. 2012). At initial presentation, the MPV of patients with non-ST-segment elevation acute myocardial infarction (AMI) is higher than controls (López-Cuenca et al. 2012). A meta-analysis published in 2010 of more than 2500 patients from 16 studies similarly demonstrated that MPV measured at the time of diagnosis was higher in patients presenting with AMI than in those without AMI (Chu et al. 2010). In this study, MPV was significantly higher regardless of whether the comparison group included studies utilizing either patients with unstable angina, with stable coronary artery disease, or those without coronary disease. Additionally, MPV was demonstrated to be associated with significantly higher risk of death or restenosis. A cross-sectional study of approximately 40,000 men and women from the Copenhagen General Population Study demonstrated an association between increasing MPV and occurrence of myocardial infarction (Klovaite et al. 2011). This association persisted even when adjusted for other cardiovascular risk factors.

Although not as strongly supported as atherothrombotic disease, platelets are increasingly recognized as a participant in venous thromboembolic disease (Schulz et al. 2013). In a population-based study of more than 25,000 subjects, MPV was found to be strongly associated with venous thromboembolism. Baseline MPV was ascertained in subjects aged 25–96 years, and these subjects were followed for 13 years (Braekkan et al. 2010). Four hundred and forty-five episodes of VTE occurred in the follow-up period with 186 of these incidents considered unprovoked. Subjects with an MPV of >9.5 fL at baseline had a 1.5-fold greater likelihood of unprovoked VTE than subjects with an MPV < 8.5 fL. This association of MPV with the incidence of VTE persisted even when adjusted for multiple additional risk factors, including age, sex, smoking, body mass index, and platelet count.

As outlined above, evidence clearly exists indicating an association between MPV and vaso-occlusive events. However, difficulties in both reproducible sampling and questions regarding its utility limit the current use of MPV as a risk stratification tool in clinical settings. It remains contested whether this association indicates a causal effect of increased MPV, meaning related to increased intrinsic platelet reactivity, or is a correlate related to other inflammatory or vascular events. Furthermore, as the etiology of these changes is uncertain, MPV at this time remains a static risk factor. Strategies to alter MPV, even if found to be useful, have yet to be determined.

Many of the first studies to investigate the etiology of variations in MPV in the general population focused on the hypothesis that platelet size heterogeneity was the result of an age-dependent decrease in size. In studies following radiolabeled platelets, both platelet size and young age were found to be positively associated with increased platelet reactivity, but these effects were independent of each either. No association between platelet size and age were identified during steady-state hematopoiesis (Thompson et al. 1983, 1984; Thompson and Jakubowski 1988). This contrasts with the non-steady-state situation observed in thrombocytopenic patients with a low platelet mass and correspondingly increased platelet production. An association between MPV, megakaryocyte size and increased megakaryocyte ploidy, and myocardial infarction has been noted (Trowbridge et al. 1984). This might suggest that intrinsic alterations in the megakaryocyte-platelet axis are causally related to both the increased MPV and its association with thrombotic events. Interestingly, recent studies indicate that alterations in the activity of the megakaryocyte scavenger receptors ABCG4 and ABCB6, regulators of cholesterol efflux and porphyrin metabolism, respectively, affect both the reactivity of circulating platelets and their thrombotic potential (Murphy et al. 2013, 2014). An intriguing hypothesis is that the megakaryocyte-platelet axis senses alterations of the vasculature through these or similar receptors and alters the platelet phenotype in response.

Age- and Maturation-Related Platelet Heterogeneity

Platelet characteristics vary throughout their lifetime in circulation. Various stages of platelet maturation have been demonstrated. To generate a platelet, mature megakaryocytes extend long, branching processes into the sinusoidal blood vessels of the bone marrow. This process of intravascular proplatelet extension by the extravascular megakaryocytes has been directly observed in murine bone marrow (Junt et al. 2007). By continued shearing of these intravascular proplatelet extensions, multiple proplatelets are thus derived from a single extravascular megakaryocyte. As these proplatelets are larger than the discoid platelet, additional stages of platelet maturation have been proposed. Fragmentation of these larger proplatelets due to shear, primarily within the lung, results in the generation of platelets (Zucker-Franklin and Philipp 2000). Another maturation stage of platelet development, the preplatelet, has recently been described (Thon et al. 2010). These circular large platelets have increased RNA content suggesting that they may be related to the young, or reticulated, platelet (Dale et al. 1995). Preplatelets convert reversibly to and from barbell-shaped platelets. Midline fission of the "barbell" results in the formation of two discoid platelets. These preplatelets may correspond to the duplicating platelet. Duplicating platelets, observed in vitro and in suspension culture, are the result of new protein translation and organelle formation from a single discoid platelet (Schwertz et al. 2010).

Proplatelet extension accounts for the large majority of newly formed platelets in non-stressed conditions. However, in response to acute platelet depletion or inflammatory stimuli, another method of platelet production accounts for a larger fraction of platelet production (Nishimura et al. 2015). Using two-photon in vivo microscopy acute megakaryocyte rupture with platelet fragmentation has recently been described in a subpopulation of megakaryocytes. This process, distinct from the slower process of proplatelet extension, results in the rapid formation of large numbers of platelets. Megakaryocyte rupture is enhanced in inflammatory conditions and acute platelet depletion and is regulated in a thrombopoietin-independent manner by the cytokine IL-1α. Interestingly, the reticulated platelet formed by this process tended to be larger in size suggesting the possibility that differential utilization of these methods of platelet production could account for some of the variations in platelet heterogeneity that have been described in human populations.

In addition to discoid platelets, platelet microparticles circulate in plasma. These cellular fragments are characterized by their size and the presence of platelet receptors on their surface. Microparticles are generated from both activated platelets and directly from megakaryocytes. When derived from activated platelets, microparticles have a short-circulating lifespan and are characterized by the presence of negatively charged phospholipids on their surface (Rand et al. 2006). These microparticles range in size from 0.1 to 1 μM in diameter with the smaller diameter particles consisting of membrane-enclosed and membrane-extruded platelet organelles. Both α-granules and isolated functional mitochondria have been demonstrated within these particles (Heijnen et al. 1999; Boudreau et al. 2014). Various functions have been attributed to these microparticle fractions. Transfer of mRNA, miRNA, and even transcription factor proteins from platelet microparticles to endothelial cells and macrophages has been demonstrated to regulate gene expression (Laffont et al. 2013, 2015; Risitano et al. 2012; Ray et al. 2008). A second class of circulating microparticles is derived directly from megakaryocytes. Distinct from activated platelet-derived microparticles, the megakaryocytic-derived microparticles express neither α-granule-derived CD62P nor the lysosomal protein LAMP-1 on their surface (Flaumenhaft et al. 2009).

Heterogeneity of Platelet Genetics

Anucleate platelets are unable to transcribe RNA from DNA. However, the machinery and templates to process and translate RNA are both present and utilized by platelets (Denis et al. 2005; Weyrich et al. 1998). Heterotypic transfer of RNA both to and from platelets has also been demonstrated. Selective transfer of specific RNAs from the megakaryocyte to the proplatelet determines the RNAs present in platelets, or the platelet transcriptome. Cecchetti et al. investigated the expression of matrix metalloproteinase (MMP)-encoding mRNAs and proteins in megakaryocytes and platelets together with their inhibitors (TIMP). Five of ten *MMP*s and one of three *TIMP*s were present in megakaryocytes but not in platelets. For example, *TIMP*-1 mRNA was abundantly expressed in both megakaryocytes and platelets, while *TIMP*-3 mRNA was highly expressed solely in megakaryocytes (Cecchetti et al. 2011). Variations of the platelet transcriptome have been demonstrated in multiple acute and chronic disease states. Compared with patients with stable coronary disease, patients with acute ST-segment elevation myocardial infarction (STEMI) had differential expression of at least 54 RNAs. Both the mRNAs for the transmembrane receptor CD69 and myeloid-related protein (MRP)-14 were increased two- to fourfold in STEMI platelets (Healy et al. 2006; Wang et al. 2014). Similarly, differential gene expression has been demonstrated in non-ST elevation acute coronary syndrome relative to those with stable cardiac disease (Colombo et al. 2011). Platelet transcriptomic differences have been associated with an increased presence of vascular disease in patients with systemic lupus erythematosus (Lood et al. 2010). Significant increases in RNAs and proteins within the type I interferon-regulated signaling pathway were found. Finally, utilization of platelets as a kind of systemic RNA surveillance mechanism has been suggested based on their ability to scavenge tumor-derived RNA-containing microvesicles. In patients with both prostate cancer and glioma, tumor-derived RNA has been detected in platelets (Nilsson et al. 2011).

Heterogeneity of Activated Platelets (Procoagulant Platelets)

Heterogeneity is also noted among platelets following platelet activation. Platelet activation is characterized by changes in both the morphologic and functional characteristics of the discoid platelet. Among these are:

A change in shape of the platelet from a rigid disc to a spread, space-filling confirmation when on a surface or an amorphous globule with filipodial extensions when observed in suspension (Hartwig 2006; Sakurai et al. 2015)

An incorporation of intracellular granules into the platelet membrane that is accompanied by the extracellular expulsion of their contents and the incorporation of the granule's membrane proteins into the plasma membrane (Ren et al. 2008)

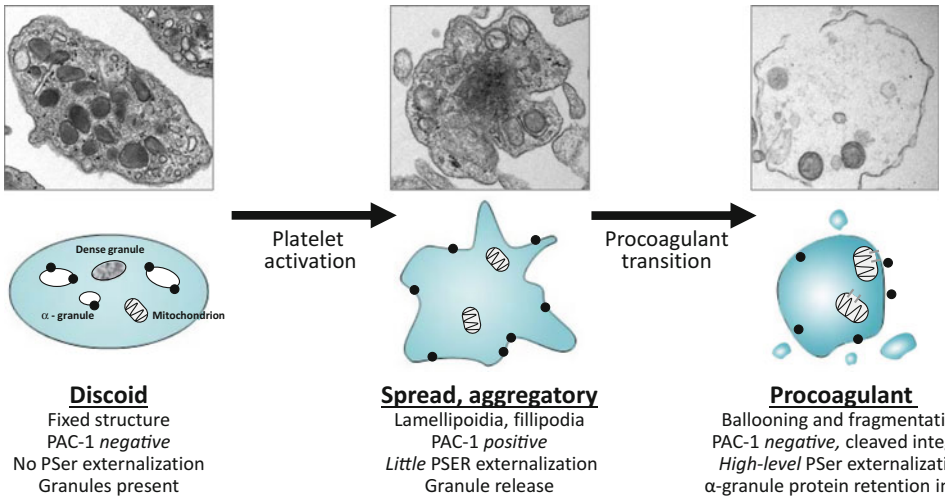

Fig. 1 Subpopulations of activated platelets. Activation of discoid platelets by platelet agonists results in extension of lamellipodia and filipodia (spreading), a change in the integrin conformation from inactive to active (PAC-1 positive), and release of alpha and dense granule. When stimulated by strong agonist(s), a subpopulation of spread platelets transition to become procoagulant platelets. These procoagulant platelets balloon and fragment; phosphatidylserine is externalized; the integrin conformation is again altered (see text), and alpha-granule proteins are retained in a cap on the platelet surface. Transmission electron micrographs (*top*) are murine platelets representative of each subpopulation prior to and following stimulation with thrombin and the GPVI agonist convulxin (Jobe SM)

A functional change in the platelet glycoprotein integrin $\alpha_{IIb}\beta_3$ that allows its interaction with soluble fibrinogen and, consequently, platelet aggregation as multiple activated platelets interact (Plow et al. 2014)

A rapid equilibration of the normally maintained plasma membrane asymmetry that results in the movement of negatively charged and procoagulant phospholipids from the internal plasmalemmal surface to the external platelet surface (Heemskerk et al. 2002)

Procoagulant Platelets and Platelet Phosphatidylserine (PSer) Externalization Not all activated platelets demonstrate all of these characteristics, and two subpopulations of activated platelets with distinctive subsets of these features can be discerned (Fig. 1). The initial experiments that described the activated platelet tended to be performed in bulk suspension. With the development of flow cytometry and epifluorescent microscopic techniques, it became clear that not all of the characteristics described above were equally distributed among activated platelets. This was true especially when these platelets had been activated by combinations of agonists or flowed over a collagen surface. Using flow cytometry in combination with labeled factor V or factor VIII, a population of platelet particles with high levels of coagulation factor binding was identified (Sims et al. 1988, 1989; Gilbert et al. 1991). Studies using annexin V, a protein with high affinity for PSer, demonstrated that the increased coagulation factor binding was due to high levels of PSer (Thiagarajan and Tait 1990, 1991; Dachary-Prigent et al. 1993).

Based on various functional and morphologic characteristics of this procoagulant subpopulation of activated platelets, a variety of names has been assigned to platelets with similar features. These include balloon-like (Heemskerk et al. 1997) or ballooned and procoagulant-spread (BAPS) (Agbani et al. 2015) platelets, convulxin and thrombin-induced-factor V (COAT-FV) platelets (Alberio et al. 2000), coated platelets (Dale 2005), sustained calcium-induced platelet morphology (SCIP) (Kulkarni and Jackson 2004), highly activated platelets (Jobe et al. 2008), and procoagulant platelets (Mattheij et al. 2013). The distinguishing characteristic of the procoagulant platelet subpopulation is the presence of high levels of PSer on the external platelet surface. PSer consists of a phospholipid tail with a negatively charged serine head. Normally, it is sequestered within the inner leaflet of the bilayer plasma membrane. In procoagulant platelets, activation of a scramblase causes this asymmetry to be rapidly lost, and PSer is externalized (Bevers et al. 1983). PSer potently facilitates the assembly of the tenase and prothrombinase coagulation complexes on the platelet surface. In the presence of small amounts of activated coagulation factors, procoagulant platelet formation results in the generation of large amounts of thrombin and the conversion of fibrinogen to fibrin (Rosing et al. 1985a, b). Externalized PSer is also recognized by macrophages and may be an important marker for the clearance of procoagulant platelets (Fadok et al. 1992).

Procoagulant Platelets and Platelet Morphology Unique morphologic characteristics distinguish the procoagulant platelet subpopulation from other activated platelets.

Among collagen-adherent platelets, procoagulant platelets are distinguished by their balloon-like appearance (Heemskerk et al. 1997; Agbani et al. 2015). This ballooning is the consequence of a rapid expansion of a small membrane extension from a non-collagen-adherent portion of the platelet surface (Agbani et al. 2015). These balloon-like platelets are also observed in hemostatic plugs, primarily in regions adjacent to collagen and on the non-luminal (vascular lumen) surface of the hemostatic plug (Wester et al. 1978; Hovig et al. 1967). Procoagulant platelets that had previously spread on fibrinogen or another extracellular surface due to integrin activation have a variant of this morphology. A central rounded platelet fragment, or balloon, is surrounded by numerous small particles distributed in the region where the platelet had previously spread (Kulkarni and Jackson 2004; Jobe et al. 2008).

Intracellularly, procoagulant platelets are marked by the absence of distinguishing organelles, including platelet granules, with the exception of occasional mitochondria seen in apposition to the cell membrane, and an electron-lucent amorphous cytoplasm (Agbani et al. 2015). In suspension, the spherical procoagulant platelet has a small portion of the plasma membrane or "cap" consisting of fibrin and other α-granule proteins (Abaeva et al. 2013). In the presence of flowing leukocytes, adherent procoagulant platelets extrude long tethers called flow-induced protrusions or FLIPRs that extend as long as 250 μm in the direction of flow (Tersteeg et al. 2014). These FLIPRs break to generate small fragments or microparticles. Similarly, ballooned and procoagulant-spread (BAPS) platelets in situations of low or minimal flow will disintegrate into a multitude of small procoagulant microvesicles that can support prothrombinase activity and fibrin formation (Agbani et al. 2015).

Procoagulant Platelet Formation and Platelet Adherence The adherence properties of the procoagulant platelet markedly differ from the spread or aggregating activated platelet. When platelets are flowed over a monolayer of spread platelets, such as fibrinogen-adherent platelets activated by a low dose of thrombin, the flowing platelets first adhere loosely to the platelet monolayer and then become stably adherent. However, on a procoagulant platelet monolayer, flowing platelets transiently interact with the monolayer, but stable interactions are decreased. The end consequence of this decrease in stable interactions is only minimal recruitment and accumulation of platelets on the procoagulant platelet surface (Kulkarni et al. 2007; Liu et al. 2013). This decreased platelet adhesiveness is also evident when a monolayer of procoagulant platelets is subjected to increasing shear stresses. Relative to spread and adherent platelets, the procoagulant platelet is less able to maintain contact with the surface as the shear stress is increased to arterial levels (Artemenko et al. 2015).

Corresponding with these functional alterations in adhesiveness, both the structure and interactions of the major adhesive receptor integrin $\alpha_{IIb}\beta_3$ are altered in procoagulant platelets. In resting platelets, $\alpha_{IIb}\beta_3$ is in an inactive state limiting its ability to engage with soluble fibrinogen and preventing platelet aggregation. Agonist stimulation results in an integrin $\alpha_{IIb}\beta_3$ extracellular conformational change and activation through the engagement of talin and kindlin with the cytoplasmic tail of the integrin β_3-subunit (Tadokoro et al. 2003; Moser et al. 2008). Integrin activation allows platelet spreading and aggregate formation. Activated integrin can be detected on the platelet surface by antibodies with specific affinity for the activated integrin $\alpha_{IIb}\beta_3$ conformation, such as PAC-1 (human) or JON/A (mouse) (Bergmeier et al. 2002). On spread and aggregating platelets, integrin $\alpha_{IIb}\beta_3$ is activated, the platelets are recognized by PAC-1, and P-selectin, a marker of platelet granule release, is expressed on the platelet surface; on procoagulant platelets, P-selectin is expressed on the platelet surface, but these platelets are not recognized by PAC-1 or JON/A (Jobe et al. 2005; Munnix et al. 2007; Dale et al. 2002). Since normal or increased levels of integrin $\alpha_{IIb}\beta_3$ are present on the procoagulant platelet surface, either the integrin is no longer in an active state or access to the integrin by the antibodies is limited. Together with this altered configuration, the cytoplasmic tail of integrin β_3 is cleaved in procoagulant platelets at a site close to the plasma membrane (Liu et al. 2013; Mattheij et al. 2013). Cleavage of this large portion of the cytoplasmic domain of integrin is predicted to limit integrin interaction with the integrin-activating proteins talin and kindlin (Du et al. 1995; Calderwood et al. 2003). Elimination of this key interaction may contribute to the observed alteration in integrin configuration and decreased platelet adherence seen in the procoagulant platelet subpopulation. Another consequence of this protein cleavage event is to limit the interaction of integrin with the platelet cytoskeleton (Schoenwaelder et al. 1997). This decreased interaction of integrin and other platelet membrane proteins, including GPIb-IX and P-selectin, with the platelet cytoskeleton may also limit the procoagulant platelet's ability to adhere in the presence of increased shear stress (Artemenko et al. 2015).

Procoagulant Platelets and α-Granule Protein Retention A distinct feature of the procoagulant platelet is the accumulation of high levels of α-granule proteins on the platelet surface (Dale 2005). Relative to spread and aggregated platelets, procoagulant platelets demonstrate increased accumulation of numerous α-granule proteins when measured

using flow cytometry, including fibrinogen, von Willebrand factor, fibronectin, and thrombospondin (Dale et al. 2002; Jobe et al. 2005). The surface association of another platelet-derived protein, tissue-factor pathway inhibitor (TFPI)-α, although not present in platelet α-granules, is similarly localized to the procoagulant platelet surface (Maroney et al. 2007). α-Granule protein retention is not evenly distributed, or coated, on the procoagulant platelet surface; instead it is localized to a single patch, or cap, on the procoagulant platelet surface (Abaeva et al. 2013).

The mechanism of increased α-granule protein retention on the procoagulant platelet surface remains uncertain. Covalent linkage of proteins released from α-granules to serotonin, which is released from dense granules, occurs on the surface of procoagulant platelets (Dale et al. 2002). Thrombospondin and fibrinogen can both interact with these serotonin-derivatized α-granule proteins. This suggests a model in which serotonin-derivatized α-granule proteins interact with both their various receptors on the platelet surface, for example, fibrin(ogen) with integrin $\alpha_{IIb}\beta_3$, and with serotonin binding sites on thrombospondin and fibrinogen to form a stable, multivalent cap on the platelet surface (Szasz and Dale 2002). However, FXIII deficiency or transglutaminase inhibition neither prevents increased α-granule protein retention nor formation of the platelet cap (Jobe et al. 2005; Abaeva et al. 2013). An alternative mechanism has also been proposed that suggests a primary role for fibrin polymerization in this process (Abaeva et al. 2013). In this proposed model, initial binding of α-granule proteins to their cognate surface receptors is followed by cell-mediated clustering of these receptors to a distinct region. Fibrin(ogen) polymerization then allows increased protein retention and stabilizes cap formation. Supporting this mechanism, cap formation was inhibited both in a patient with altered formation of fibrin polymers and by pharmacologic inhibition of fibrin. Formation of the protein cap may be essential in the retention of procoagulant platelets within the aggregate as inhibition of cap formation blocked the incorporation of procoagulant platelets into aggregates.

A recent study using a fluorescent transglutaminase substrate suggests the presence of heterogeneity of platelet surface protein modifications even within the procoagulant platelet subpopulation (Mattheij et al. 2015). Using this probe, transglutaminase activity, identified as factor XIII, was found to be isolated to a subpopulation of procoagulant platelets. Increased surface transglutaminase activity was a delayed event on this subpopulation of procoagulant platelets and followed both platelet PSer externalization and changes in integrin conformation. In the absence of FXIII activity, platelet localization of fibrin formation to procoagulant platelet surfaces did not occur in flow conditions. The authors thus suggest a role for procoagulant-mediated transglutaminase activity in fibrin localization within forming thrombi.

Procoagulant Platelets and Leukocyte Recruitment and Activation Following the establishment of hemostasis, a key component of the response to a break in vascular integrity is the recruitment of leukocytes to the site of injury. Soon after hemostatic plug formation in vivo, platelets and neutrophils can be observed in close apposition at the edge of the thrombus (Hovig et al. 1968; Jorgensen et al. 1967; Wester et al. 1979). This neutrophil recruitment prevents bacterial colonization of the wound. Dysregulated neutrophil recruitment by platelets, however, may contribute to the pathogenesis of multiple inflammatory processes, including lung injury (Zarbock et al. 2006), asthma (Pitchford et al. 2003), atherosclerosis (May et al. 2008), ischemia-reperfusion injury (Lefer et al. 1998), and sepsis (Singer et al. 2006). Activated platelets, both aggregating and procoagulant, facilitate neutrophil recruitment through granule release and elaboration of P-selectin on the platelet surface. Engagement of P-selectin by PSGL-1 on the leukocyte surface can tether leukocytes at the site of injury (Diacovo et al. 1996; Moore et al. 1995). Generation of a cytokine gradient through release of the α-granule protein NAP-2 (neutrophil-activating peptide-2) can direct leukocyte transmigration through the thrombus (Ghasemzadeh et al. 2013). Consolidation of neutrophil recruitment is then dependent on activation of neutrophil β_2-containing integrins, such as Mac-1 and LFA1 (Diacovo et al. 1996), in a process facilitated by platelet-activating factor (PAF) and certain cytokines generated by activated platelets (Ostrovsky et al. 1998).

Procoagulant platelets may play a unique role in mediating neutrophil activation and firm adhesion of platelets to neutrophils both within the hemostatic plug and in circulating monocyte/neutrophil microparticle complexes. Neutrophil interactions have been reported with “lysed” or balloon-like platelets in both in vitro and in vivo studies of clot morphology (Jorgensen et al. 1967; Hagberg et al. 1998). Studies evaluating the interaction of neutrophils with an adherent platelet monolayer have demonstrated that stable neutrophil adhesion and spreading, unlike platelet tethering, is closely correlated with the number of procoagulant platelets in the adherent platelet monolayer (Kulkarni et al. 2007). Synthesis of PAF was closely correlated with an increase in procoagulant platelet formation, and inhibition of the neutrophil PAF receptor blunted neutrophil activation and spreading on immobilized, spread platelets. A role for procoagulant-derived PAF is further suggested by studies examining neutrophil recruitment and migration in newly generated platelet thrombi (Ghasemzadeh et al. 2013). PAF receptor antagonists substantially reduced neutrophil Mac-1 activation and directed leukocyte migration to the core of the

hemostatic plug. Monocyte and neutrophil interactions with procoagulant-generated flow-induced protrusions (FLIPRs) can similarly result in leukocyte activation (Tersteeg et al. 2014). Mac-1 activation, L-selectin shedding (another marker of activation), and the formation of leukocyte/platelet microparticle aggregates were all dependent on FLIPR formation when monocytes or neutrophils were flowed over adherent platelets.

Molecular Mechanisms of Procoagulant Platelet Formation The molecular mechanisms driving procoagulant platelet formation are distinct. Strong agonist stimulation drives procoagulant platelet formation. High doses of thrombin, adhesion to collagen, or stimulation with multiple agonists have all been demonstrated to initiate procoagulant platelet formation (Rosing et al. 1985a; Alberio et al. 2000; Heemskerk et al. 1997). Other weaker stimuli that can potentiate formation by stronger agonists include von Willebrand factor, shear stress, and ADP (Kulkarni and Jackson 2004; Leytin et al. 2004; Delaney et al. 2014). Among soluble agonists, the combination of a collagen mimetic, which stimulates intracellular signaling via the GPVI receptor, and thrombin, which stimulates intracellular signaling through the G-protein-coupled protease-activated receptors (PARs), is the most potent stimulus (Jobe et al. 2005; Alberio et al. 2000). In flowing whole blood, collagen alone can stimulate procoagulant platelet formation (Heemskerk et al. 1997). However, even with the most potent of physiologic agonists, not all platelets become procoagulant (Dale 2005; Schoenwaelder et al. 1997).

Sustained elevation of cytoplasmic calcium levels is an essential trigger for procoagulant platelet formation in collagen and dual-stimulated platelets (Smeets et al. 1993; Dachary-Prigent et al. 1995; Choo et al. 2012). Store-operated calcium entry of extracellular calcium through the STIM1/Orai1 complex contributes to the sustained elevations of calcium seen in dual-stimulated platelets (Gilio et al. 2010). Coincident signaling through the GPVI and thrombin-activated signaling pathways also activates cation entry via the transient receptor potential channels TRPC3 and TRPC6 that drive further elevation of cytoplasmic calcium levels (Harper et al. 2013).

Another unique feature of procoagulant platelets is the role of mitochondria. The mitochondrial permeability transition pore (mPTP) is a multiprotein complex within the inner mitochondrial membrane, the opening of which results in a large non-gated pore (Halestrap and Richardson 2015). Cyclophilin D is a mitochondrial peptidylprolyl isomerase that is an important positive regulator of mPTP opening (Baines et al. 2005). In the absence of cyclophilin D activity, either through genetic deletion in mice or via pharmacologic inhibition in humans, platelet mPTP formation and, subsequently, procoagulant platelet formation are markedly impaired (Jobe et al. 2008; Remenyi et al. 2005). Platelet granule release and integrin activation are unaffected in cyclophilin D's absence, but the morphologic and functional changes associated with procoagulant platelets, including PSer externalization and integrin activation, are attenuated. An increase in mitochondrial calcium levels can cause mPTP opening, and it has been proposed that entry of cytosolic calcium through the mitochondrial calcium uniporter (MCU) may trigger this event (Choo et al. 2012).

Intracellular events downstream of mPTP formation and cytosolic calcium elevation mediate the morphologic and functional changes associated with procoagulant platelet formation (Brooks et al. 2007; van Kruchten et al. 2013). Two separate pathways mediate the changes in adherence and platelet PSer exposure (Briedé et al. 1999; Liu et al. 2013). Elevated cytosolic calcium levels and other, yet to be identified, mitochondrial-mediated changes trigger massive activation of intracellular calpain (Liu et al. 2013; Choo et al. 2012). This results in the cleavage of cytoskeletal proteins and major platelet adhesive receptors, including integrin $\alpha_{IIb}\beta_3$ (Schoenwaelder et al. 1997). Inhibition of calpain activity prevents both cellular rounding and the alterations in platelet adherence (Schoenwaelder et al. 1997; Kulkarni and Jackson 2004; Mattheij et al. 2013; Artemenko et al. 2015). PSer exposure is unaffected by inhibition of calpain activity (Ivanciu et al. 2014). TMEM16F, also known as anoctamin-6, regulates PSer exposure in procoagulant platelets (Suzuki et al. 2010; Yang et al. 2012; Fujii et al. 2015). TMEM16F's absence results in an absence of PSer exposure in procoagulant platelets but only partially inhibits the morphologic and adhesive changes observed in procoagulant platelets (Mattheij et al. 2013).

Necrotic Versus Apoptotic Procoagulant Platelets Procoagulant platelet formation, regulated by cyclophilin D and calcium, is reminiscent of cellular necrosis (Zong and Thompson 2006). Platelet rounding and phosphatidylserine exposure are also observed in platelets exposed to Bcl-2 antagonists (Mason et al. 2007; Zhang et al. 2007; Vogler et al. 2011). Unlike in procoagulant platelets formed as a result of platelet activation, formation of these PSer-exposing platelets requires neither sustained calcium elevation nor cyclophilin D (Schoenwaelder et al. 2009). Instead, PSer exposure by Bcl-2 antagonists requires the proapoptotic Bcl-2 family proteins Bax and Bak in a process more reminiscent of cellular apoptosis than cellular necrosis. While cyclophilin D-regulated necrotic platelet formation regulates activated platelet phenotype, this process of Bax-Bak-dependent platelet apoptosis is a key determinant of platelet lifespan (Mason et al. 2007). In the absence of the pro-survival Bcl-2

protein Bcl-xL, a key cellular inhibitor of Bax and Bak, platelet lifespan is severely shortened resulting in thrombocytopenia. This thrombocytopenia is also seen in vivo following treatment with a Bcl-xL inhibitor (Zhang et al. 2007).

Platelet Heterogeneity Within the Hemostatic Plug and Thrombus

Within a forming thrombus or hemostatic plug discoid platelets, aggregated and spread platelets and procoagulant platelets are all observed. Electron microscopy studies and in vivo fluorescent microscopy demonstrate distinct distributions of each of these types of activated platelet (Fig. 2). Electron microscopy studies of the newly formed hemostatic plug demonstrate a distinct architecture of the hemostatic plug (Wester et al. 1978; Hovig et al. 1967). In the center of the plug partially to fully degranulated platelets form a consolidated aggregate. Platelets are in close contact; granules, mitochondria, and other structures can be visualized; varying degrees of pseudopod formation are evident; and little fibrin is interspersed between the platelets. In contrast to the appearance of platelets in the center of the plug, platelets adjacent to collagen and platelets at the periphery of the hemostatic plug were electron lucent and had lost their internal structure, including both granules and mitochondria, appeared to have gaps in their membranes, and were typically located adjacent to fibrin strands. Based on this empty, electron-lucent appearance, some investigators have compared their appearance to balloons. Over time, progressively more platelets within the center of the thrombus also transitioned to a balloon morphology and become surrounded by fibrin (Hovig et al. 1968; Wester et al. 1979). Arterial thrombus formation following disruption of the endothelial layer demonstrates patches of both interdigitated and degranulated platelets intermixed with discoid platelets with intact platelet granules (Hechler et al. 2010; Jorgensen et al. 1967).

Dynamic in vivo imaging of hemostatic plug and thrombus formation has provided further insight into the evolution of this distinct platelet architecture. Typically, in these in vivo imaging studies, a focus of endothelial injury is induced using a laser, and the extent and depth of injury are controlled by altering the laser intensity. A characteristic pattern of platelet activation is observed when a penetrating laser injury with a small amount of bleeding is caused or when a small external puncture is formed (Ivanciu and Stalker 2015). Platelet accumulation begins almost immediately after injury. Given the instability of this original platelet accumulation, presumably these are discoid platelets. Discoid platelets have been observed as the first accumulating platelets at sites of endothelial injury, especially in the presence of shear gradients (Jackson shear gradient and Heemskerk geometries) (Nesbitt et al. 2009; Westein et al. 2013). Within the first minute after injury, a core of activated platelets is gradually formed at the site of injury (Stalker et al. 2013). Using fluorescent antibodies and sensors, the molecular nature of this core can be determined. Fibrin slowly accumulates both within and immediately inferior to the platelet core accompanied by increased thrombin activity (Welsh et al. 2012; Ivanciu et al. 2014); platelets within the core are P-selectin positive indicating

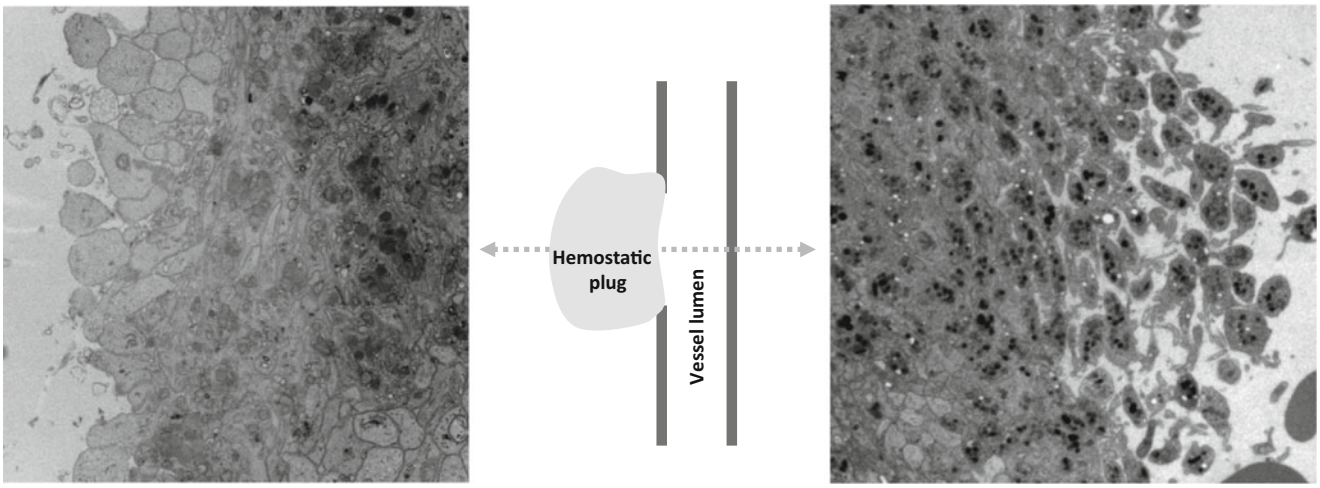

Fig. 2 Heterogeneity within the hemostatic plug. Representative transmission electron micrographs of section of a murine, mesenteric hemostatic plug 5 min after injury. *Left*. Section from the non-luminal periphery of the hemostatic plug. Electron-lucent platelets (balloon platelets) rim the hemostatic plug with consolidation of the hemostatic plug toward the core. On the luminal periphery, loosely-associated and granule-containing discoid platelets aggregate next to a consolidated, plug of platelets (Jobe SM)

that granule release has occurred (Stalker et al. 2013); and the platelets are densely packed limiting solute transport (Welsh et al. 2014). Superior to the core, a shell of platelets is observed, consisting of less densely packed platelets without evidence of platelet granule release. Platelets within this shell are transients within the thrombus with constant dissolution and reaccumulation of single platelets and loosely connected aggregates (Stalker et al. 2013).

How and whether procoagulant platelets accumulate in the thrombus or hemostatic plug appears to vary depending on the nature of the injury. In electron microscopy studies, balloon platelets are distributed as described above. Markers of phosphatidylserine exposure, however, cannot be used in fixed platelets, and their utility in in vivo studies is not clear. Using annexin V to label procoagulant platelets and labeled fibrinogen, distinct patches of procoagulant platelets were noted spread among fibrinogen-binding aggregated platelets in ferric chloride-damaged and ferric chloride-ligated carotid arteries (Munnix et al. 2007; Berny et al. 2010). Similarly, small patches of procoagulant platelets distributed throughout the thrombus were seen in a model of venous thrombosis. Procoagulant platelets within the thrombus can also be identified using 4-[N-(S-glutathionylacetyl)amino] phenylarsonous acid (GSAO), a marker of cell death. Accumulation of GSAO-labeled platelets varied depending on the mechanism of injury (Hua et al. 2015). In a model of injury with minimal endothelial damage and collagen exposure, only very little, if any, accumulation of procoagulant platelets was noted. In an occlusive model of carotid injury induced by ferric chloride, procoagulant platelets in association with fibrin were noted throughout the occlusive thrombus.

Take-Home Messages

- Interindividual differences in mean platelet volume (MPV) have been correlated with multiple pathologies, including an association of increased MPV with increased risk of both arterial and venous thrombosis.
- Within an individual, platelet characteristics vary throughout their lifetime in vivo. In addition to discoid platelets, preplatelets, proplatelets, and microparticles circulate in plasma.
- The anucleate platelet contains genetic information in the form of both RNA and miRNA. Differential transfer of genetic material from the megakaryocyte to the platelet, and from heterologous cells in circulation, can alter gene expression in platelets.
- Distinct aggregatory and procoagulant subpopulations occur within collagen-adherent platelets and strongly stimulated platelets in suspension.
- Procoagulant platelets have distinct morphologic and functional characteristics including:
  - Externalization of high levels of phosphatidylserine and increased procoagulant activity
  - A rounded, balloon-like morphology
  - Impaired adhesive characteristics associated with changes in integrin $\alpha_{IIb}\beta_3$ structure and function
  - Proinflammatory recruitment of neutrophils
- Distinct intracellular signaling mechanisms regulate procoagulant platelet formation.
- In vivo, platelet heterogeneity is a prominent characteristic of hemostatic plugs. Discoid, aggregatory, and balloon-like platelets can be distinguished by electron microscopy.

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Regulation of Platelet Adhesion Receptors

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Abstract

Platelet adhesion to subendothelial matrices and cohesion to other platelets is essential for hemostasis, but recent studies have revealed that platelet adhesive interactions may also generate cross-talk with other cell types, including leukocytes, tumor cells, and microbial pathogens. In so doing, these adhesive interactions may impact a variety of protective or disease-promoting processes, and they are mediated by platelet adhesion receptors from several gene families, most notably integrins, as exemplified by the platelet-restricted integrin, $\alpha_{IIb}\beta_3$. During hemostasis, multiple brakes and controls on platelet adhesion receptors prevent pathological thrombosis. Thus, threshold levels of excitatory stimuli must be surpassed and sufficient to drive intracellular signaling for full activation of integrin receptors. Stimuli for primary hemostasis derive from released or generated soluble agonists, vascular matrices exposed by vessel injury, or by hydrodynamic shear stress. While the non-integrin adhesion receptor, GPIb-V-IX, is critical for platelet adhesion to matrix von Willebrand factor (VWF) during hemostasis and is a mediator of platelet aggregation through interactions with VWF at pathologically high shear stresses in atherosclerotic arteries, finely regulated interactions of $\alpha_{IIb}\beta_3$ with fibrinogen and other Arg-Gly-Asp (RGD)-containing ligands are required for platelet aggregation in hemostatic thrombi. In this chapter we present an overview of classical and nonclassical regulation of platelet adhesion receptors and associated signaling pathways, with a principle focus on platelet signaling pathways that converge on and regulate $\alpha_{IIb}\beta_3$.

Introduction

Platelets are adhesion machines. Indeed, every known biological function or pathological role of platelets, from hemostasis-thrombosis (Varga-Szabo et al. 2008), to regulation of aspects of embryonic development (Echtler et al. 2010; Watson et al. 2014), to participation in innate and adaptive immunity (Herter et al. 2014; Kapur et al. 2015), and to tumor promotion (Labelle et al. 2011) involves platelet adhesion receptors in one way or another. As epitomized by integrin $\alpha_{IIb}\beta_3$, the adhesion and signaling functions of these plasma membrane receptors are tightly regulated to enable rapid platelet hemostatic responses to vascular injury and to avoid excessive platelet responses leading to pathological thrombosis, including myocardial infarction and stroke (Bennett 2015; Coller 2015; Plow et al. 2014; Shattil et al. 2010). Here we review what is known about how platelet adhesion receptor function is regulated, a subject relevant to other chapters that discuss broader aspects of platelet function in health and disease.

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Regulation of Platelet Integrins

Platelets express several members of the integrin superfamily (Hynes 2002): $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$. All are type I transmembrane α- and β-heterodimers, each subunit consisting of a relatively large extracellular domain, a single-span transmembrane domain, and a short cytoplasmic domain of ~20–47 amino acids. $\alpha_{IIb}\beta_3$ is highly expressed, with ~80,000 copies on the surface of resting platelets, a number so high that individual $\alpha_{IIb}\beta_3$ heterodimers are estimated to be spaced a mere ~125 Å apart (Wagner et al. 1996). There is a smaller internal pool of $\alpha_{IIb}\beta_3$ expressed on granule membranes that can become surface expressed during granule secretion (Woods et al. 1986).

The principal extracellular ligands for $\alpha_{IIb}\beta_3$ are fibrinogen and VWF, although under appropriate experimental conditions, the receptor can engage other RGD-containing ligands such as vitronectin and fibronectin (Plow et al. 1985). Binding of $\alpha_{IIb}\beta_3$ to soluble plasma fibrinogen (Bennett and Vilaire 1979) or VWF (Jackson 2007; Ruggeri 2002) supports platelet aggregation because these multivalent ligands can engage in trans-interactions, thereby functioning as bridges between $\alpha_{IIb}\beta_3$ receptors on adjacent platelets. Initial platelet adhesion to damaged vessels involves the interaction of immobilized VWF with the non-integrin platelet adhesion receptor, GP Ib-V-IX (discussed below), and immobilized RGD ligands can support platelet adhesion and spreading via $\alpha_{IIb}\beta_3$ (Jirouskova et al. 2007).

The principal ligands for platelet $\alpha_V\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta1$ are vitronectin (Felding-Habermann and Cheresh 1993), collagen (Nieswandt and Watson 2003a), fibronectin (McCarty et al. 2004), and laminin (Schaff et al. 2013), respectively. Compared to $\alpha_{IIb}\beta_3$, expression of these integrins in platelets is Lilliputian, e.g., being less than 1–2 % that of $\alpha_{IIb}\beta_3$. This does not mean they have no functional role, but their roles are likely relatively minor under physiologic conditions. For example, studies of mouse or human platelets indicate that $\alpha_2\beta_1$ plays a secondary role as a collagen receptor, with GP VI playing the dominant signaling role (Nieswandt and Watson 2003a).

Platelets contain RNA and the machinery for translation of RNA into proteins, but they do not contain their own DNA (Schubert et al. 2014). Consequently, the function of platelet integrins is regulated primarily at the posttranslational level, a process studied most intensively for $\alpha_{IIb}\beta_3$ (Bennett 2015; Plow et al. 2014; Shattil et al. 2010). Regulation of $\alpha_{IIb}\beta_3$ is often divided arbitrarily into two phases: **inside-out signaling**, which controls integrin activation state and ligand binding, and **outside-in signaling**, which is triggered by ligand binding leading to the generation of inward signals that promote further platelet responses, including activation of additional integrins, cytoskeletal rearrangements, platelet secretion, and the development of platelet procoagulant activity (Coller and Shattil 2008).

Positive Regulators of Integrin Activation

"Classical" Fluid-Phase Excitatory Platelet Agonists

Platelets are equipped with multiple routes of activation that together unleash an avalanche of complex signaling events to effect platelet shape change and exocytosis from platelet alpha and dense granules (Golebiewska and Poole 2015) as well as activation of pathways that convert $\alpha_{IIb}\beta_3$ and other integrins to a high-affinity state. A wide array of molecular species is involved in this inside-out signaling process, including kinases, phosphatases, isomerases, adaptor proteins, and those controlling lipid trafficking (Brass et al. 2013; Furie and Flaumenhaft 2014; O'Donnell et al. 2014). The concerted action of multiple agonist receptors ensures that initial responses to individual agonists are amplified to support stable, irreversible platelet aggregation and thrombus formation to effect hemostasis.

The classical excitatory platelet agonists engage G protein-coupled receptors (GPCR) in the plasma membrane that signal through heterotrimeric G proteins, the latter consisting of α-, β-, and γ-subunits. Ligand binding to GPCRs induces exchange of GTP for GDP on Gα and its separation from the Gβγ subunits, each then promoting downstream activation or repression of distinct pathways (Gurbel et al. 2015; Offermanns 2006). Classical GPCR agonists include (1) ADP released from hemolyzed red blood cells or from dense granules during platelet secretion; (2) thromboxane A2 generated de novo following release of arachidonic acid from membrane phospholipids and its conversion to prostaglandin endoperoxides by cyclooxygenase-1 (Samuelsson et al. 1978); (3) thrombin, a product of the coagulation cascade; and (4) epinephrine, a circulating hormone (Brass et al. 2013). Under normal circumstances, none of these agonists is available to circulating platelets at sufficient concentrations to surmount normal restraints to platelet activation, arguing that a minimal threshold trigger must exist to initiate the release or generation of these soluble agonists at effective microenvironmental concentrations.

ADP is an example of a so-called "weak" agonist that by itself promotes little secretion and submaximal $\alpha_{IIb}\beta_3$ activation, yet is central to full platelet activation initiated by other agonists. ADP binds three purinergic receptors on platelets, P2Y1 and P2Y12 (Cattaneo 2015) and P2X1 (Jones et al. 2014). P2Y1 and P2Y12 are needed for a full aggregation response to ADP, whereas Ca^{2+} influx through P2X1 amplifies ADP-induced calcium signaling triggered by

P2Y1. P2Y1 associates with Gαq to regulate platelet shape change and granule secretion, and platelets from P2Y1 knockout mice fail to aggregate in response to low concentrations of ADP and respond only partially to other agonists (Leon et al. 1999). P2Y12 associates with Gαi2 to repress ADP-mediated generation of cAMP, a negative regulator of platelet activation (Smolenski 2012). P2Y12 also promotes signaling through phosphatidyl inositide 3-OH kinase (PI3-K) independently of protein kinase C (PKC) (Kauffenstein et al. 2001), and supports the slow, irreversible phase of platelet aggregation (Jarvis et al. 2000). Src family kinases (SFKs) also appear to play a role in Gi-mediated signaling, as ADP- and epinephrine-induced platelet aggregation is decreased in the presence of SFK inhibitors (Nash et al. 2010). Mice lacking P2Y12 show decreased $\alpha_{IIb}\beta_3$ activation, reduced adhesion to VWF under flow, and small, unstable thrombi on damaged mesenteric arteries (Andre et al. 2003). In a recent in vivo thrombosis model, Stalker et al. found that thrombi were hierarchically organized, with a core of highly activated platelets and a shell of less-activated platelets, whose size and stability were strongly dependent on P2Y12 and Gi signaling (Stalker et al. 2013). Defects in ADP receptors in patients mimic those seen in P2Y1 and P2Y12 knockout mice and produce a mild hemorrhagic phenotype (Cattaneo and Gachet 1999; Nurden et al. 1995; Cattaneo 2011). Moreover, P2Y12 antagonists have proven to be extremely useful in the prophylaxis and treatment of acute coronary syndromes (Rollini et al. 2015).

Thromboxane A2 is a clinically important platelet agonist, as suggested by the widespread use of aspirin in the primary prevention of cardiovascular events. Aspirin inhibits COX1 and thromboxane production in platelets. Thromboxane A2 binds the TP receptor and signals through Gq and G12/13 leading to shape change and intracellular calcium mobilization (Offermanns 2006). Full platelet aggregation in response to thromboxane A2 is dependent on released ADP (Paul et al. 1999). TP knockout mice have prolonged bleeding times (Thomas et al. 1998). **Thrombin** is a "strong" agonist that binds with high affinity to the non-GPCR, GPIb-V-IX, as well as to the protease-activated GPCR receptors, PAR1 and PAR4 in humans and PAR3 and PAR4 in mice. Thrombin cleaves the extracellular N-terminus of PAR1 and PAR4 to expose the novel tethered ligands, SFLLRN and AYPGKF, respectively (Coughlin 2005). Platelet activation through PAR receptors is mediated by Gαq and Gα13 (Brass et al. 2013). **Epinephrine** binds the α2A adrenergic receptor (Kaywin et al. 1978). It is a weak agonist that functions primarily to potentiate platelet aggregation induced by other agonists, and it can overcome P2Y12 blockade through Gαz signaling (Lova et al. 2011).

"Nonclassical" Excitatory Agonists

Matrix-Associated Platelet Agonists Functioning Through Hemostatic Pathways

Platelet adhesion receptors are hardwired to promote and amplify mechanisms that enhance the activation of integrins, particularly $\alpha_{IIb}\beta_3$, for stable platelet adhesion to immobilized ligands or for binding of soluble integrin ligands. Resting platelets under static, non-flow conditions can adhere via integrins to immobilized ligands, e.g., to immobilized fibrinogen via $\alpha_{IIb}\beta_3$ (Savage et al. 1992), to collagen via $\alpha_2\beta_1$ (Nieswandt and Watson 2003a), to laminin via $\alpha_6\beta_1$ (Geberhiwot et al. 1999), and to fibronectin via $\alpha_5\beta_1$ (McCarty et al. 2004). These interactions can trigger outside-in signaling pathways that serve as feedback activation loops for upregulation of integrin affinity and/or clustering (Ruggeri and Mendolicchio 2007). Additionally, fibrinogen and vitronectin may become immobilized on extracellular matrices upon vessel injury, bind to platelets through β_3 integrins, and contribute to outside-in integrin signaling and thrombus formation. Under shear flow conditions, where hydrodynamic forces can easily sweep away loosely attached platelets, a priori activation of integrins for attachment to fibronectin and VWF is needed (Beumer et al. 1994; Savage et al. 1996).

The non-integrin platelet adhesion receptors, GPVI and GPIb-V-IX, recognize exposed matrix collagen and VWF, respectively, and in so doing signal to activate integrins through either of two associated co-receptors, FcRγ and FcγRIIA, that contain immunoreceptor tyrosine-based activation motifs (ITAM) with tandem YXXL sequences (Canobbio et al. 2001; Falati et al. 1999). In addition, GPIb-V-IX can signal independently to $\alpha_{IIb}\beta_3$ (Kasirer-Friede et al. 2004). Under flow conditions, GPVI and GPIb-V-IX serve to bridge the temporary anchoring of platelets onto matrices with stable adhesion through activation of $\alpha_{IIb}\beta_3$. At arterial shear rates, platelet attachment to collagen is mediated through the VWF/GPIb-V-IX axis (discussed below), and at lower shear, collagen-induced signaling to $\alpha_{IIb}\beta_3$ is primarily through GPVI (Lecut et al. 2004). Studies utilizing various inhibitors and genetically modified mice have helped to identify proteins downstream of GPIb-V-IX and GPVI and their Fc co-receptors that relay signals involved in integrin activation. These include protein tyrosine kinases (e.g., SFKs, Syk, Tec family kinases), serine-threonine kinases (e.g., phospholipase C (PLC), PKC, PI3-K), and adaptor proteins (e.g., SLP-76, ADAP) (Bergmeier and Stefanini 2013; Bryckaert et al. 2015).

$\alpha_V\beta_3$ is expressed in platelets in small numbers relative to $\alpha_{IIb}\beta_3$ and can engage vitronectin, adenovirus penton base (Wickham et al. 1993) and other ligands, such as osteopontin

(Bennett et al. 1997), which may be present in atherosclerotic plaques. In experimental cell assays, $\alpha_V\beta_3$ can undergo activation similar to that of $\alpha_{IIb}\beta_3$ in response to ADP (Kasirer-Friede et al. 2006). In vitro experiments have suggested that long VWF strings secreted from Weibel-Palade bodies of activated endothelial cells can be sequestered by endothelial surface-expressed $\alpha_V\beta_3$ for subsequent recognition by platelet GPIb-V-IX (Huang et al. 2009). Under in vivo conditions, however, VWF strings are observed only in the venous vasculature and appear to be independent of P-selectin and β_3 integrin (De Ceunynck et al. 2013).

Platelet Agonists Functioning Through Non-hemostatic Pathways

It is becoming increasingly evident that platelets do not function exclusively in the realm of hemostasis but are actively involved in communication with and co-regulation of multiple cell types in the processes of host defense, innate immunity, atherosclerosis, airway and liver disease, and cancer progression (Franco et al. 2015; McFadyen and Kaplan 2015). These interactions may result in mutual cellular activation and often utilize platelet adhesion receptors that normally operate in hemostasis.

Platelet-immune cell interactions are manifold. GPIbα on matrix-attached platelets may facilitate recruitment of neutrophils to vessel walls through binding to leukocyte integrin $\alpha_M\beta_2$ (Mac-1) (Ehlers et al. 2003; Simon et al. 2000). In addition, microparticles bearing active Mac-1 and released from stimulated neutrophils can bind to platelets via GPIbα and promote platelet P-selectin surface expression and $\alpha_{IIb}\beta_3$ activation (Pluskota et al. 2008). Dying neutrophils may also release extracellular traps (NETS) that are part of their antibacterial defense mechanisms (Martinod and Wagner 2014) and contain DNA, histones, and alkaline proteins. NETS can enmesh platelets and provide a NET scaffold for thrombus growth (Fuchs et al. 2010). While VWF has been found closely associated with DNA (Grassle et al. 2014), platelets may directly interact with histones H3 and H4 in part through platelet Toll-like receptors (TLR)'s 2 and 4 (Semeraro et al. 2011). Furthermore, several histones have the ability to act as substrates for platelet adhesion and spreading and to stimulate fibrinogen binding to $\alpha_{IIb}\beta_3$. Platelet activation triggered by histones is mediated by ERK, Akt, and p38 (Carestia et al. 2013).

Galectins (Gal-) are a family of highly conserved lectins, which recognize cell surface and matrix β-galactosidases. They may be found in soluble or immobilized form in a wide variety of tissues and are implicated in immune and tumor responses (Schattner and Rabinovich 2013). Galectins recognize the carbohydrate moieties of $\alpha_{IIb}\beta_3$ and GPIb-V-IX, resulting in mild increases in platelet adhesion, aggregation, and spreading (Schattner 2014).

A chemically distinct nonclassical integrin activator is the CXCR6 receptor, recently identified in platelets. The CXC motif ligand 16 (CXL16), found in atherosclerotic plaques, or purified in vitro, can serve as an adhesive ligand for platelets under shear flow (Meyer Dos Santos et al. 2015) and promote $\alpha_{IIb}\beta_3$ activation and platelet secretion through PI3-K dependent pathways (Borst et al. 2012).

Microbial pathogens constitute a large class of potential platelet modulators, as both bacteria and viruses have been implicated under various experimental conditions in direct binding to GPIb-V-IX, $\alpha_{IIb}\beta_3$, and other receptors on platelets, leading to $\alpha_{IIb}\beta_3$ activation, fibrinogen binding, and alpha-granule secretion (Hamzeh-Cognasse et al. 2015). A novel mechanism for dengue virus invasion of platelets was recently demonstrated that utilizes platelet surface-expressed DC-SIGN and heparin sulfate for entry and replication, thereby sheltering the virus from patrolling immune cells (Simon et al. 2015). The FcγRIIA receptor may also be hijacked by pathogens to breach and activate platelets (Boilard et al. 2014; Arman et al. 2014). Platelets express TLRs 2, 7, and 9, which harbor pathogen recognition sequences. Certain pathogens can activate platelets (Cox et al. 2011), perhaps helping to explain in part the thrombotic consequence of sepsis (de Stoppelaar et al. 2014).

Principal Signaling Pathways Downstream of Agonist Receptors Involved in Activation of $\alpha_{IIb}\beta_3$

G proteins connect to an array of signaling molecules, and agonist pathway specificity results, in part, by distinct isoform expression of signaling intermediates (Min and Abrams 2013; O'Donnell et al. 2014).

Platelets contain at least five isoforms of PLC, including PLCβ and PLCγ2. PLCβ promotes signaling through G protein βγ subunits, Gq and G12/13, while PLCγ2 is implicated downstream of protein tyrosine kinases. Both isoforms hydrolyze the polar head group of membrane phosphatidyl inositol 4,5 biphosphate (PtdIns (4,5) P2) to yield diacylglycerol (DAG) and inositol (1,4,5) triphosphate (IP3), which releases calcium from dense tubules (Brass and Joseph 1985). Influx of extracellular calcium through the Orai-1 channel (Braun et al. 2009, 2011) further drives cyclical intracellular calcium elevations. Multiple cytoplasmic and cytoskeletal proteins affecting integrin activation harbor calcium binding sites and become activated in response to calcium transients, including regulatory proteins such as calpain and calmodulin (Elsaraj and Bhullar 2008), the classical and nonclassical members of the PKC family, and CALDAG-GEFI which, as discussed below, functions as a guanine nucleotide exchange factor for Rap1b (Crittenden et al. 2004; Cifuni et al. 2008).

Protein kinase C family members are serine/threonine kinases that may positively or negatively regulate $\alpha_{IIb}\beta_3$

and platelet activation, depending on the agonist and PKC isoform involved (Harper and Poole 2010). Tyrosine phosphorylation of PKC may be induced by thrombin and collagen (Hall et al. 2007; Murugappan et al. 2005) and can affect its activity, localization (Crosby and Poole 2003; Pula et al. 2005), or association with receptors for activated C-kinase (RACK) proteins (Schechtman and Mochly-Rosen 2001). PKCα is best known for phosphorylating substrates that regulate alpha- and dense-granule secretion, including SNAPs, SNAREs, and VAMPs, and for regulating integrin activation by promoting Rap1-mediated activation of talin (Cifuni et al. 2008; Gilio et al. 2010; Han et al. 2006; Strehl et al. 2007). PKCβ and PKCθ appear to significantly modulate outside-in $\alpha_{IIb}\beta_3$ signaling (Buensuceso et al. 2005; Soriani et al. 2006). Although the specific PKC subtypes have not been identified, pan-PKC inhibitors were used to identify novel roles for PKC in promoting 12-lipoxygenase-induced $\alpha_{IIb}\beta_3$ activation (Yeung et al. 2012) and in maintaining proteasomal activity (Nayak et al. 2011) that sustains platelet aggregation by low-dose thrombin or GPIb-V-IX (Gupta et al. 2014).

PI-3Ks phosphorylate the 3-OH group of the inositol ring of phosphatidyl inositols (PI), which can then bind specific PH and Src homology-2 (SH2) domains in proteins (Min and Abrams 2013; O'Donnell et al. 2014). At least four PI-3K isoforms are expressed in platelets, (Guidetti et al. 2015). The two best characterized are the Class Ia member, p85/p110 PI-3Kβ, and the Class Ib member, p110 PI-3Kγ (Rittenhouse 1996; Zhang et al. 1996), with PI3-Kβ thought to play the predominant role in platelet inside-out signaling to $\alpha_{IIb}\beta_3$ downstream of GPCRs and ITAM-bearing receptors (Guidetti et al. 2015). However, platelet signaling may involve other PI3-K isoforms, such as PI3-Kα acting downstream of the insulin-like growth factor receptor (Hers 2007), or PI3-Kγ acting downstream of ADP stimulation (Hirsch et al. 2001). One of the principal effectors of PI3-K in platelets is Akt (Protein kinase B, PKB), with three platelet isoforms expressed, each supporting platelet aggregation in an agonist-selective manner (Laurent et al. 2014; Moroi and Watson 2015). PI3-K helps recruit PDK1 to phosphorylate Akt at the platelet membrane (Dangelmaier et al. 2014). Mice deficient in Akt1 and Akt2 have impaired thrombin-induced fibrinogen binding and thrombus formation in vivo (Woulfe et al. 2004), and Akt3 mice exhibit defects in thrombus growth that may be due to ineffective deactivation of the Akt platelet inhibitory target, glycogen synthase kinase-3b (GSK3β) (O'Brien et al. 2011). The potential importance of Akt activation and GSK3β inhibition is supported by recent studies showing that deacetylation of Akt lysine residues by sirtuin 2 (SIRT2) can modulate its capacity to phosphorylate and thereby deactivate GSK3β to allow full platelet aggregation (Moscardo et al. 2015).

While signaling pathways downstream of platelet excitatory receptors are multiple and often interacting, major attention has focused on Rap1b, a membrane-anchored Ras family GTPase, because it has been implicated in integrin activation in many cell types, including platelets (Fig. 1, Table 1) (Gloerich and Bos 2011). Knockout of Rap1b in mice is associated with reduced, but not absent, platelet fibrinogen binding and aggregation in response to both GPRC- or non-GPRC-coupled agonists (Chrzanowska-Wodnicka et al. 2005).

Rap1 is inactive as a pro-aggregatory signaling intermediate when bound to GDP in unstimulated platelets. The Rap1-GDP state is promoted by a Rap1 GAP, RASA3. Upon platelet stimulation through the $P2Y_{12}$ ADP receptor and activation of PI3K, the inhibitory effect of RASA3 on Rap1b is blocked (Stefanini et al. 2015). In addition, platelet activation mediated by ADP and other agonists leads to the generation of ionized free calcium and diacylglycerol, and the former activates a Rap1 GEF, CalDAG-GEFI (RASGRP2), that converts Rap1-GDP to active Rap1-GTP (Crittenden et al. 2004; Eto et al. 2002; Larson et al. 2003). CalDAG-GEFI overexpression enhances agonist-induced fibrinogen binding to $\alpha_{IIb}\beta_3$ in murine megakaryocytes (Eto et al. 2002), and CalDAG-GEFI knockout mouse platelets show reduced aggregation in response to agonists (Crittenden et al. 2004). Moreover, a mutation in CalDAG-GEFI (cG742T) in man is associated with impaired activation of Rap1 and $\alpha_{IIb}\beta_3$ in platelets, although leukocyte adhesion is normal (Canault et al. 2014). Bergmeier and co-workers suggest that fast but reversible Rap1 activation in platelets is dependent on calcium and CalDAG-GEFI, while sustained Rap1 activation requires signaling through P2Y12, PKC, and PI3K (Cifuni et al. 2008; Stefanini et al. 2015).

One Rap1 effector that is expressed in platelets is RIAM, and its role in integrin activation was studied initially in leukocytes and in model cell systems (Han et al. 2006; Lafuente et al. 2004). In a CHO cell system frequently utilized to study ectopically-expressed human $\alpha_{IIb}\beta_3$, sh-RNA mediated knockdown of Rap1 or RIAM impaired PAR1 thrombin receptor-mediated activation of $\alpha_{IIb}\beta_3$, and similar results were obtained in embryonic stem cell-derived murine megakaryocytes (Kahner et al. 2012; Watanabe et al. 2008). One current model is that platelet agonists trigger activation of Rap1, which then recruits RIAM to the membrane. Since RIAM can bind to talin directly (Chang et al. 2014; Lee et al. 2009), this provides talin direct access to the integrin β_3 cytoplasmic tail, thus triggering activation of $\alpha_{IIb}\beta_3$ (Shattil et al. 2010).

Recent work indicates that this model of inside-out $\alpha_{IIb}\beta_3$ signaling is incomplete. First, there must be Rap1-independent routes to $\alpha_{IIb}\beta_3$ activation, since as mentioned, agonist-induced fibrinogen binding to and aggregation of

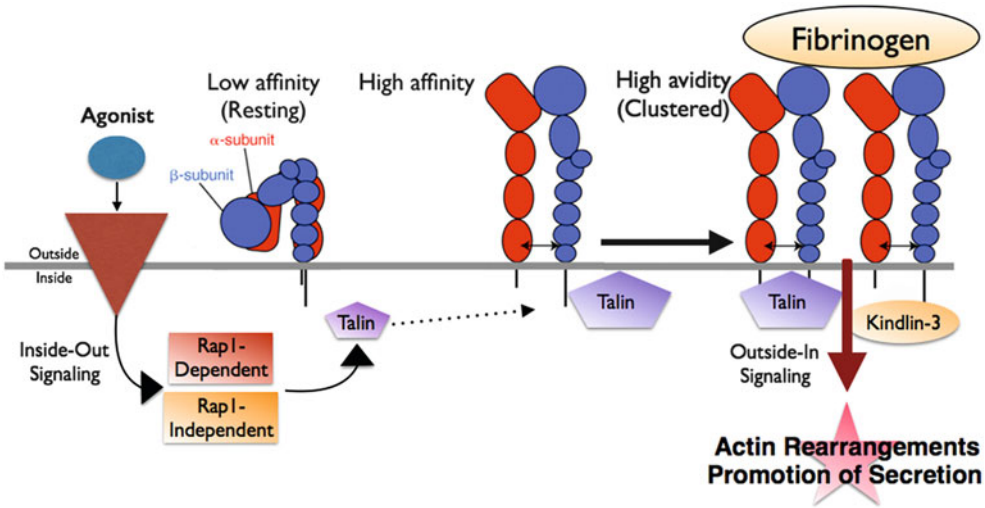

Fig. 1 Schematic overview of signaling impacting the most abundant platelet adhesion receptor, $\alpha_{IIb}\beta_3$. In resting platelets, $\alpha_{IIb}\beta_3$ is in a low-affinity state. Upon platelet activation by a range of excitatory agonists (Table 1), inside-out signaling is triggered, and $\alpha_{IIb}\beta_3$ is converted through propagated conformational changes starting at the α_{IIb} and β_3 cytoplasmic tails and transmembrane domains to a high-affinity state capable of productive interactions with soluble fibrinogen and other cognate agonists, including von Willebrand factor and fibronectin. Inside-out signals converge on poorly defined Rap1-dependent or Rap1-independent effectors that act on talin, resulting in its recruitment to the plasma membrane and its interaction with the integrin β_3 cytoplasmic tail to induce the high-affinity state of $\alpha_{IIb}\beta_3$. The *double arrows* indicate that the transmembrane domains separate during activation. In addition, kindlin interacts with the β_3 cytoplasmic tail to promote $\alpha_{IIb}\beta_3$ clustering within the plane of the plasma membrane, thereby enhancing $\alpha_{IIb}\beta_3$ avidity for its matrix ligands. Fibrinogen binding to high-affinity-clustered $\alpha_{IIb}\beta_3$ triggers outside-in signaling mediated by talin, kindlin-3, and a host of other proteins, including Src, Syk, and FAK protein tyrosine kinases to help promote additional responses during platelet activation, including actin rearrangements, and platelet spreading, granule secretion, and development of platelet procoagulant activity

Table 1 Summary of excitatory agonists regulating activation of integrin $\alpha_{IIb}\beta_3$

Excitatory agonists of $\alpha_{IIb}\beta_3$ activation					
Classical		Nonclassical		Nonclassical	
Soluble agonists	Platelet receptors	Principal matrix ligands	Platelet receptor	Cellular stimulation	Platelet receptors
ADP	P2X1, P2Y1, P2Y12	VWF, fibrinogen, fibronectin	$\alpha_{IIb}\beta_3$	Tumor cells	GPIb-V-IX
Thrombin	GPIbα, PAR1, PAR4	Vitronectin, osteopontin	$\alpha_V\beta_3$	Leukocytes	GPIb-V-IX, P-selectin
Epinephrine	α2A	Collagen	$\alpha_2\beta_1$	Endothelial cells	GPIb-V-IX, P-selectin
TXA2	TP	Fibronectin	$\alpha_5\beta_1$	Pathogens	GPIb-V-IX, FcR's,
		VWF	GPIb-V-IX		$\alpha_{IIb}\beta_3$, TLR's, DC-SIGN
		Collagen, Laminin	GPVI		

Classical soluble agonists in the extracellular space bind their cognate platelet receptors to initiate inside-out signaling. Nonclassical signaling pathways to $\alpha_{IIb}\beta_3$ may be initiated in response to platelet adhesion receptor binding to subendothelial matrix ligands (VWF, laminin, fibronectin, collagen) or to immobilized fibrinogen or vitronectin deposited from plasma. Alternatively, platelet interactions with multiple cell types may engage platelet receptors to promote and amplify signaling to $\alpha_{IIb}\beta_3$. These stimulatory pathways may utilize different isoform-specific signaling intermediates of the same families, such as PI-3K and PLC, but ultimately unite to converge on integrin proximal regulatory molecules, such as talin and kindlin-3

Rap1b-null murine platelets is decreased, but not abolished (Fig. 1) (Chrzanowska-Wodnicka et al. 2005). Rap1-independent routes to $\alpha_{IIb}\beta_3$ activation remain to be clarified, but likely include yet-to-be-identified substrates of PKC. Second, RIAM is dispensible for $\alpha_{IIb}\beta_3$ activation in mouse platelets (Stritt et al. 2015), indicating that another, as yet unidentified, Rap1b effector must participate in integrin activation in these cells. Regardless, both Rap1-dependent and Rap1-independent pathways eventually must merge in proximity to the $\alpha_{IIb}\beta_3$ cytoplasmic tails to stimulate $\alpha_{IIb}\beta_3$ activation by the integrin cytoplasmic tail-binding proteins, talin and kindlin-3.

Integrin-Proximal Regulatory Events

Talin-1 is a 215 kDa adaptor protein whose functions in platelets include promotion of integrin activation, linkage of integrins to the actin cytoskeleton, and promotion of clot retraction (Plow et al. 2014; Shattil et al. 2010). A related gene product, talin-2, is not expressed in platelets. Talin is composed of an N-terminal head domain containing a

FERM domain with four sub-domains (F0–F3) and a long C-terminal rod domain. It can interact directly with integrin cytoplasmic tails and with actin and other proteins (Roberts and Critchley 2009). In unstimulated platelets, a large pool of talin is in the cytoplasm, presumably in the form of auto-inhibited, antiparallel homodimers, and in response to platelet stimulation with thrombin, talin becomes phosphorylated on Ser and/or Thr residues and translocates to the plasma membrane in a process that does not require $\alpha_{IIb}\beta_3$ (Bertagnolli et al. 1993). As discussed above, Rap1 and its effector(s) are involved in this recruitment process (Fig. 1). It is still not known precisely how talin is converted from its auto-inhibited conformation to a conformation that enables it to interact with binding partners, such as integrins, vinculin, and actin, although talin interaction with PIP_2 may be important in this regard (Wang 2012). In addition, the role of talin phosphorylation (Ratnikov et al. 2005) as well as the precise mechanism of Rap1-independent talin recruitment to the plasma membrane remain to be determined.

Talin interactions of known importance to $\alpha_{IIb}\beta_3$ activation include that of the talin head domain with negatively charged plasma membrane phospholipids, such as PIP_2, and with the β_3 cytoplasmic tail (Fig. 1) (Garcia-Alvarez et al. 2003; Ginsberg 2014; Wegener et al. 2007). Two regions of the β_3 tail are involved in this interaction. One is centered at the first of two NPXY sequences that are present in the tail, and it provides most of the free energy for the interaction with the F3 sub-domain of the talin head. The other involves a juxtamembrane region of the β_3 tail, and this interaction is directly required for inducing conformational changes that lead to full-blown $\alpha_{IIb}\beta_3$ activation and fibrinogen binding (Ginsberg 2014; Petrich et al. 2007; Ye et al. 2014). Talin also plays a necessary role in outside-in $\alpha_{IIb}\beta_3$ signaling leading to the spreading of fibrinogen-adherent platelets and fibrin clot retraction (Haling et al. 2011) and in promoting the adhesive functions of platelet β_1 integrins (Petrich et al. 2007).

Over the past decade, crystallographic and electron microscopic studies of the extracellular domain of $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$, coupled with NMR studies of the α_{IIb} and β_3 transmembrane and cytoplasmic domains, have provided a working model of talin-dependent $\alpha_{IIb}\beta_3$ activation (Coller 2015; Ginsberg 2014; Lau et al. 2009; Luo et al. 2007; Park et al. 2015; Xiao et al. 2004; Zhu et al. 2010). Key elements include a transmembrane allosteric rearrangement of the α_{IIb} and β_3 subunits whereby agonist-triggered inside-out signals and the binding of talin to the β_3 tail disrupt α_{IIb} and β_3 transmembrane interactions to induce a change in tilt angle of the β3 transmembrane domain with respect to α_{IIb}. In the inactive integrin, the transmembrane domains of α_{IIb} and β_3 feature a so-called outer membrane clasp promoted by glycine packing interactions and an inner membrane clasp promoted by hydrophobic stacking of amino acid side chains and by formation of a salt bridge involving α_{IIb} (R995) and β_3 (D723). In this conformation, the side chain of a positively charged β_3 residue (K716) orients to snorkel back toward the membrane boundary, where it associates with a negatively charged phospholipid group. This results in a 25° tilt of the β_3 alpha helix that embeds the β_3 transmembrane helix at an angle through the membrane (Kim et al. 2011). Conformational activation occurs when talin binds to the β_3 tail NPXY sequence and to acidic membrane lipid groups, leading to disruption of the (D723)/β_3 and (R995)/α_{IIb} salt bridge, and formation of a new salt bridge between β_3 and talin. The resulting increase in crossing angle of the β_3 transmembrane domain disturbs the outer membrane clasp leading to propagation of conformational changes to the extracellular domains of $\alpha_{IIb}\beta_3$ that foster ligand binding (Ginsberg 2014; Iwamoto and Calderwood 2015). The changes include conversion of $\alpha_{IIb}\beta_3$ from a bent to an upright conformation, a piston-like downward axial displacement of the β_3 I-like domain C-terminal helix resulting in a swing-out of the β_3 hybrid and PSI domains from the I-like domain, and a conversion of the N-terminal ligand-binding headpiece from closed to open. The β_3 I-like domain contains three metal ion sites termed MIDAS, ADMIDAS, and SyMBS that play important roles in the activation and ligand-binding processes (Coller 2015). Current models of $\alpha_{IIb}\beta_3$ activation may be of general relevance to other integrins.

Studies of purified talin added to phospholipid nanodiscs containing $\alpha_{IIb}\beta_3$ indicate that talin binding is sufficient to initiate $\alpha_{IIb}\beta_3$ activation, at least in vitro (Ye et al. 2010). However, talin is not sufficient for normal agonist-induced fibrinogen binding to murine or human platelets, and at least one other molecular adaptor protein, kindlin-3, is involved (Kuijpers et al. 2009; Malinin et al. 2009; Moser et al. 2008; Svensson et al. 2009).

Kindlin-3 is a ~76 kDa protein expressed almost exclusively in hematopoietic cells, including platelets (Malinin et al. 2010; Moser et al. 2009a). It is a member of the kindlin family of adaptor molecules that includes kindlin-1 and kindlin-2. Its modular structure includes a FERM domain near the C-terminus which can engage several integrin β tails, including β_3, and in which the F2 sub-domain is uniquely split by a PH domain. Some kindlin family members have been shown to contain binding sites for other proteins, including integrin-linked kinase, migfilin, c-Src, and RACK1 (Feng et al. 2012; Malinin et al. 2010; Moser et al. 2009b; Qu et al. 2014). The extent to which kindlin-3 can engage any of these proteins in platelets remains to be determined.

Given the necessary and sufficient role for talin in $\alpha_{IIb}\beta_3$ activation in membrane nanodiscs in vitro, the inability of stimulated mouse or human platelets deficient in kindlin-3 to

bind fibrinogen and aggregate normally provides an additional layer of complexity to the $\alpha_{IIb}\beta_3$ activation story. Kindlin-3 deficiency also impairs leukocyte integrin function, and in humans this combined platelet and leukocyte defect is associated with serious recurrent infections and bleeding and is referred to as Leukocyte Adhesion Deficiency III (or LAD I variant) (Etzioni 2014; Kuijpers et al. 2009; Malinin et al. 2009; Svensson et al. 2009). Talin and kindlin-3 binding to the β_3 cytoplasmic tail is not mutually exclusive, and kindlin depletion or excess does not affect agonist-induced talin recruitment to $\alpha_{IIb}\beta_3$ (Kahner et al. 2012). One idea based on recent experimental work is that talin functions primarily to induce conformational change and affinity modulation of $\alpha_{IIb}\beta_3$, while kindlin functions primarily to promote $\alpha_{IIb}\beta_3$ clustering within the plane of the plasma membrane (avidity modulation) (Fig. 1) (Ye et al. 2013, 2014). It is also possible that kindlin-3 interacts with other proteins to promote platelet activation and further studies are needed to address this issue.

ADAP, also known as FYB for Fyn binding protein, is another hematopoietic-specific adaptor involved in integrin-mediated leukocyte and platelet adhesion, although the mechanism of ADAP's effects on integrins in these two cell types differs (Wang and Rudd 2008; Kasirer-Friede et al. 2014). Based on studies with human platelets and gene-targeted murine platelets, ADAP functions downstream of $\alpha_2\beta_1$ (Jarvis et al. 2012) and both upstream and downstream of $\alpha_{IIb}\beta_3$ (Kasirer-Friede et al. 2006, 2010,). Its upstream function is suggested by the finding that fibrinogen binding to $\alpha_{IIb}\beta_3$ and platelet aggregation in response to agonists is reduced in ADAP-null murine platelets (Kasirer-Friede et al. 2007). However, in contrast to the severe $\alpha_{IIb}\beta_3$ activation defect in talin- or kindlin-3-deficient mouse platelets, the defect in ADAP-null platelets is dependent on the agonist used and is generally partial. Biochemical studies indicate that ADAP can interact with talin and kindlin-3 in vitro, although it is not known if these interactions are direct (Kasirer-Friede et al. 2014). These proteins also co-localize with each other in platelets, and in ADAP-deficient platelets, there is reduced co-localization of talin with $\alpha_{IIb}\beta_3$, coupled with reduced irreversible fibrinogen binding to $\alpha_{IIb}\beta_3$. ADAP can also associate with an $\alpha_{IIb}\beta_3$-talin complex in a CHO cell expression system and enable kindlin-3 to promote agonist-dependent ligand binding to $\alpha_{IIb}\beta_3$ in these cells (Kasirer-Friede et al. 2014).

A homozygous nonsense mutation in ADAP in five members of an Arab-Christian family has been associated with thrombocytopenia with small platelets, with the remaining platelets exhibiting increased basal binding of an activation-dependent antibody to $\alpha_{IIb}\beta_3$, but reduced antibody binding in response to ADP (Levin et al. 2015). An Iraqi family with recessive thrombocytopenia with small platelets exhibiting a different homozygous deletion in Fyb has been reported, but platelet function was not studied (Hamamy et al. 2014). Taken together, these studies point to a role for ADAP in platelet biogenesis and in inside-out $\alpha_{IIb}\beta_3$ signaling mediated by talin and kindlin-3.

$G\alpha_{13}$ and $\alpha_{IIb}\beta_3$ function. As illustrated above by talin, kindlin-3, and ADAP, intracellular proteins that interact directly with $\alpha_{IIb}\beta_3$ may function in both inside-out and outside-in signaling. An interesting twist to this phenomenon is suggested by recent studies of the $G\alpha_{13}$ G protein subunit. On the one hand, the switch region 2 of $G\alpha_{13}$ can interact directly with the talin head domain in a calcium-dependent manner, leading the authors to suggest a role for this interaction in $\alpha_{IIb}\beta_3$ inside-out signaling (Srinivasan et al. 2015). On the other hand, $G\alpha_{13}$ functions downstream of PAR1 in platelets to activate a Rho GEF, leading to the activation of RhoA and platelet shape change (Brass et al. 2013). The potential involvement of fibrinogen-bound $\alpha_{IIb}\beta_3$ in mediating cytoskeletal events downstream of Gα13 is illustrated by the demonstration that Gα13 can interact directly with the β_3 cytoplasmic tail, and interference with this interaction in mouse platelets blocks outside-in $\alpha_{IIb}\beta_3$ signaling and platelet spreading on fibrinogen (Gong et al. 2010).

Negative Regulators of Integrin Activation

Proteins that Prevent Basal Activation of Integrins

Platelet activation may normally be downregulated by several mechanisms in order to prevent pathological thrombosis. These include (1) production of PGI_2 by endothelial cells leading to PGI_2 receptor-mediated production of cyclic AMP in platelets (Moncada and Vane 1981; Raslan and Naseem 2014; Weksler 1982); (2) production of nitric oxide within endothelial cells, leukocytes, and platelets leading to increased platelet cyclic GMP (Naseem and Riba 2008); and (3) enzymatic reduction in levels of extracellular ADP by endothelial cell surface-expressed CD39 (Marcus et al. 2005). cAMP and cGMP activate protein kinase A (PKA) and protein kinase G (PKG), respectively, which through their serine kinase activity serve to attenuate platelet responses. Two important substrates of PKA are the IP3 receptor, leading to prohibition of intracellular calcium elevations, and CALDAG-GEFI, the principal GTP exchange factor for Rap1b in platelets. PKA and PKG also directly phosphorylate Rap1b and VASP at distinct sites, to maintain platelets in a resting state. Platelet phosphodiesterases further regulate the impact of NO and PGI2 by degrading the phosphodiester bond in cAMP and cGMP and thus countering their effects (Smolenski 2012; Jones et al. 2012). A deficiency in one or more of these negative regulatory mechanisms could in theory increase the basal state of $\alpha_{IIb}\beta_3$ activation in platelets, although there is little direct evidence for this in humans. A number of cyclic AMP and

cyclic GMP substrates in platelets have been identified, and their effects on platelet signaling mechanisms demonstrated (e.g. inhibition of calcium fluxes). The precise roles of many of these substrates in dampening the process of $\alpha_{IIb}\beta_3$ activation are unclear.

Proteins that Promote Integrin Deactivation by Their Presence

Given that the interaction of talin and kindlin-3 with the β_3 tail promotes $\alpha_{IIb}\beta_3$ activation, it is reasonable to assume that there may be tail-interacting proteins in platelets that inhibit integrin activation. The α_{IIb} cytoplasmic tail is not a completely passive player in inside-out signaling (Liu et al. 2015b), and several proteins can bind to the highly conserved membrane-proximal GFFKR region of α integrin cytoplasmic tails to dampen integrin activation, at least in nucleated cells. Examples include MDGI and Sharpin, the latter hypothesized to indirectly block talin interaction with the integrin β_1 tail (Bouvard et al. 2013). Proteins such as filamin that bind to β cytoplasmic tails may also dampen integrin activation by blocking tail interactions with talin or kindlins (Bouvard et al. 2013; Liu et al. 2015a; Truong et al. 2015). Additional studies are required to determine the extent to which these proteins are expressed and function in platelets as negative regulators of $\alpha_{IIb}\beta_3$.

Regulation of Non-integrin Platelet Adhesion Receptors

Non-integrin platelet adhesion receptors include the highly glycosylated single-pass transmembrane proteins, GPVI, GPIb-V-IX, and CLEC-2 (Ozaki et al. 2013). Under physiological conditions, the adhesive activities of GPVI and GPIb-V-IX are controlled, in part, by de novo expression of their cognate ligands (collagen and VWF, respectively) on injured vessels or ruptured plaque, and during embryonic development by platelet CLEC-2 encounters with podoplanin expressed on the surface of lymphatic endothelial cells. Localization to lipid rafts or receptor oligomerization may further regulate the function of these particular adhesion receptors (Ozaki et al. 2013). As an example, greater than one third of GPVI exists as dimers on resting platelets, and this increases to up to 50 % following ligation or platelet thrombin activation (Jung et al. 2012), supporting the idea that the dimerized state may be the functional GPVI unit for binding to collagen (Horii et al. 2006) and possibly to laminin (Inoue et al. 2006) and fibrin (Mammadova-Bach et al. 2015). In order to selectively study GPVI signaling independently of integrin $\alpha_2\beta_1$, which also binds collagen, collagen-related peptide (CRP) (Asselin et al. 1997) and the snake-venom convulxin (CVX) (Francischetti et al. 1997) are often used in vitro to engage GPVI. GPVI is solely expressed in platelets and megakaryocytes and therefore is considered to be a potential therapeutic hemostatic target.

CLEC-2 is a member of the Dectin family of hemITAM receptors that contain only a single YXXL motif (Ozaki et al. 2013), but compensates by signaling to Syk from the dimerized state (Hughes et al. 2010). Although signaling by CLEC-2 to activate integrins is transduced through coupled tyrosine kinase cascades, as for GPVI, the architecture and hierarchy of signaling pathways may vary (Manne et al. 2015). Interaction of CLEC-2 in platelets and podoplanin in lymphatic endothelial cells is necessary for the separation of lymphatic and blood vessels (Ozaki et al. 2013). Recently, platelet CLEC-2 has been shown to associate with S100A13 in vascular smooth muscle cells and contribute to thrombus formation (Inoue et al. 2015).

Although studies in vitro and in mice implicate GPVI/Fc receptors and CLEC-2 in hemostasis and thrombosis, patients with mutations in GPVI suffer from only a mild bleeding phenotype (Arthur et al. 2007; Dumont et al. 2009), and mutations of CLEC-2 in patients are awaiting discovery. The case for GPIb-V-IX is very different. Patients lacking or with mutated GPIb-V-IX suffer from Bernard-Soulier syndrome and can exhibit a severe bleeding phenotype. The consequences of disruption of GPIB-V-IX in mice in fact overshadow those of its principal hemostatic ligand, VWF (Bergmeier et al. 2006), as noted by an absolute GPIb-V-IX requirement for platelet incorporation into arterial thrombi, but residual thrombus growth in the absence of VWF (Ni et al. 2000). This may reflect interactions of GPIb-V-IX with a range of additional binding partners and their respective contributions to thrombosis (Gardiner and Andrews 2014).

The subunits of the GPIb-V-IX complex are present in a molecular ratio of 2 GPIb α: 4 GPIb β: 2 GPIX: 1 GPV (Li and Emsley 2013; Ruggeri and Mendolicchio 2007). In contrast to GPIb-V-IX's ability to directly bind immobilized VWF at low to high shear rates, regulation of receptor and/or ligand must occur to permit platelet interactions with soluble VWF and to drive downstream signaling to activate $\alpha_{IIb}\beta_3$. While hydrodynamic stretching of multimeric VWF can cause unraveling of constituent subunits (Siedlecki et al. 1996), GPIb-V-IX binding epitopes may not be exposed and additional modifications may be needed (Miyata et al. 1996). It is likely that conformational changes in GPIb-V-IX also occur that regulate binding to VWF, as activating mutations in GPIbα, mostly in the partially ordered β-switch region in the C-terminal cap, result in the spontaneous platelet binding to VWF seen in patients with platelet-type VWD (Huizinga et al. 2002; Weiss et al. 1982). A shift in the macroglycosidic stalk region of GPIb-V-IX also appears to occur following ligation by a VWF A1 domain fragment, producing a novel epitope recognized by a recently developed antibody against this region (Zhang

et al. 2015). These and other studies have led to the model of a force-induced super affinity state that integrates several transitions in GPIb-V-IX/VWF bond behavior, from slip, flex to catch bonds as a function of applied stress, and provide a structural basis for their interactions under flow (Blenner et al. 2014). GPIb-V-IX function may be further regulated by the cytoskeletal protein, filamin A, which binds to residues 540–590 in GPIbα, and by the signaling protein, 14-3-3-ζ, which binds to residues 605–610. The 14-3-3-ζ interaction is hypothesized to repress GPIb-V-IX stimulation of $\alpha_{IIb}\beta_3$-mediated platelet spreading through Rac1 and Cdc42 (Bialkowska et al. 2003). At high shear stresses exceeding 90 dyn/cm^2, 14-3-3-ζ may dissociate from GPIb-V-IX to allow recruitment of filamin and enhance anchoring to the cytoskeleton (Cranmer et al. 2011). Several of these observations were made in heterologous cells and need to be confirmed in platelets.

Several mechanisms exist to downregulate the function of the non-integrin adhesion receptors. Immune receptor-based inhibitory motif (ITIM) domain-containing proteins such as PECAM-1 or G6b-B that recruit phosphatases (Jones et al. 2009), or inhibit Src and Syk signaling (Mori et al. 2008), may dampen responses propagated by ITAM containing receptors. Functional adhesion receptors may be reduced on the platelet surface by internalization after ligand binding (Lorenz et al. 2015; Michelson et al. 1991; Rabie et al. 2007) or by proteolysis, although thrombin cleavage of GPV may actually enhance its interaction with GPIbα (Dong et al. 1997; Phillips and Agin 1977). Metalloproteinases of the ADAM family have recently been identified as key regulators of surface expression of the non-integrin platelet adhesion receptors: ADAM 17 can cleave GPIbα and GPVI, and ADAM 10 can cleave GPVI and CLEC-2 (Gardiner et al. 2007; Al-Tamimi et al. 2012). The shed receptor domains are released into the circulation (Gawaz et al. 2014), and plasma levels appear to correlate with cardiovascular events (Qiao et al. 2010). Our understanding of how the relevant metalloproteinases are regulated in vivo is incomplete.

Perspective

Many, if not most, adhesion receptors in platelets are regulated at the posttranslational level, integrin $\alpha_{IIb}\beta_3$ being the poster child because it is the most abundant and has been studied most. Lessons learned about the regulation of $\alpha_{IIb}\beta_3$ in platelets resulted in the development of the first anti-integrin therapeutics in the 1990s with important indications in cardiovascular medicine. Candidate therapeutics targeted to non-integrin platelet adhesion receptors, particularly GP Ib-V-IX, are the subject of current studies, and they might eventually prove useful in some clinical settings. Continued investigation into how platelet adhesion receptors are regulated promises to provide additional opportunities for therapeutic intervention in thrombosis and to clarify the molecular basis of defects in platelet function in individuals with poorly understood bleeding diatheses.

Take-Home Messages

- Platelets are equipped with integrin (section "Regulation of Platelet Integrins") and non-integrin platelet adhesion receptors (section "Regulation of Non-integrin Platelet Adhesion Receptors") that together function to secure platelets to damaged endothelium under a wide range of hemodynamic stress conditions, corresponding to the arterial and venous circulation. Non-integrin platelet adhesion receptors often establish the first contacts with vascular matrices, but require integrins for stable adhesion.
- Platelets respond to classical soluble excitatory agonists, as well as to stimulation through platelet adhesion receptors bound to vascular matrices (Table 1). Agonist-associated signaling through G-protein-coupled or tyrosine-coupled receptors promotes integrin activation through *inside-out* signaling pathways to mediate stable platelet adhesion, and aggregation through cross-bridging of ligands bound to αIIbβ3 integrin.
- Intermediate signaling networks are vast and complex and include hundreds of proteins including adapter proteins, kinases, phosphatases, phospholipid mediators, and calcium (section "Principal Signaling Pathways Downstream of Agonist Receptors Involved in Activation of αIIbβ3").
- Signaling pathways converge on integrin-proximal proteins such as talin and kindlin-3 that activate the integrin by strategically binding to integrin cytoplasmic tails, provoking conformational changes in integrin extracellular domains and receptor clustering (section "Integrin-Proximal Regulatory Events").
- Ligand binding to αIIbβ3 induces inward signals (*outside-in*) to promote cytoskeletal remodeling leading to ligand stabilization, platelet spreading, and subsequent clot retraction.

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The Platelet Glycoprotein Ib-IX-V Complex

José A. López

Abstract

The glycoprotein (GP) Ib-IX-V complex is an abundant plasma membrane receptor that is expressed almost exclusively on platelets and megakaryocytes. It is highly specialized to carry out its most import important function: the initial adhesion of platelets to sites of vessel wall injury in regions of the vasculature where the wall shear stresses are very high. It does this by binding the plasma and vessel wall protein von Willebrand factor. Failure of these hemostatic mechanisms because of deficiency or dysfunction of either ligand or receptor greatly increases the risk for pathological bleeding. The complex also has other important functions, including facilitating the response of platelets to the important agonist thrombin, and involvement in inflammatory processes by interacting with neutrophil and monocyte integrin $\alpha_M\beta_2$. In this chapter, the structure, synthesis, and functions of the GPIb-IX-V complex are discussed.

Introduction

The GPIb-IX-V complex is a unique membrane adhesion complex whose existence was first inferred with the description and early characterization of a rare bleeding disorder characterized by moderate thrombocytopenia, a prolonged bleeding time, and very large platelets (Bernard and Soulier 1948). This disorder, now known as the Bernard–Soulier syndrome in honor of the two French physicians who first described it, illustrates several characteristics of the complex: its vital role in platelet adhesion, its association with the cytoskeleton, and its role in platelet formation. In this chapter, I will attempt to summarize a portion of the vast literature related to this receptor.

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GPIb-IX-V Complex Structure and Biosynthesis

The term GPIb-IX-V complex generally refers to a complex of four primary polypeptides: GPIbα, GPIbβ, GPIX, and GPV, present on the platelet membrane in a stoichiometry of 2:4:2:1, respectively (Fig. 1). Other membrane proteins of platelets are known to associate with this complex, but for the purposes of this chapter, they are not considered part of the complex because they have been shown to function independently and not require the complex for their expression.

Each of the GPIb-IX-V complex polypeptides is produced by its own gene, and each is a type I membrane protein with a single hydrophobic transmembrane domain (López 1994). Although the genes are not clustered in the genome, they are similar in being compact, each gene having only one intron, except the *GPIX* gene, which has two (López et al. 1998). Each of the genes also contains the entire protein-coding sequence within a single exon, with the exception of *GPIBB*, which has a small intron interrupting the coding sequence only ten base pairs after the first base of the initiator methionine. The proteins these genes encode are also related, each belonging to the large,

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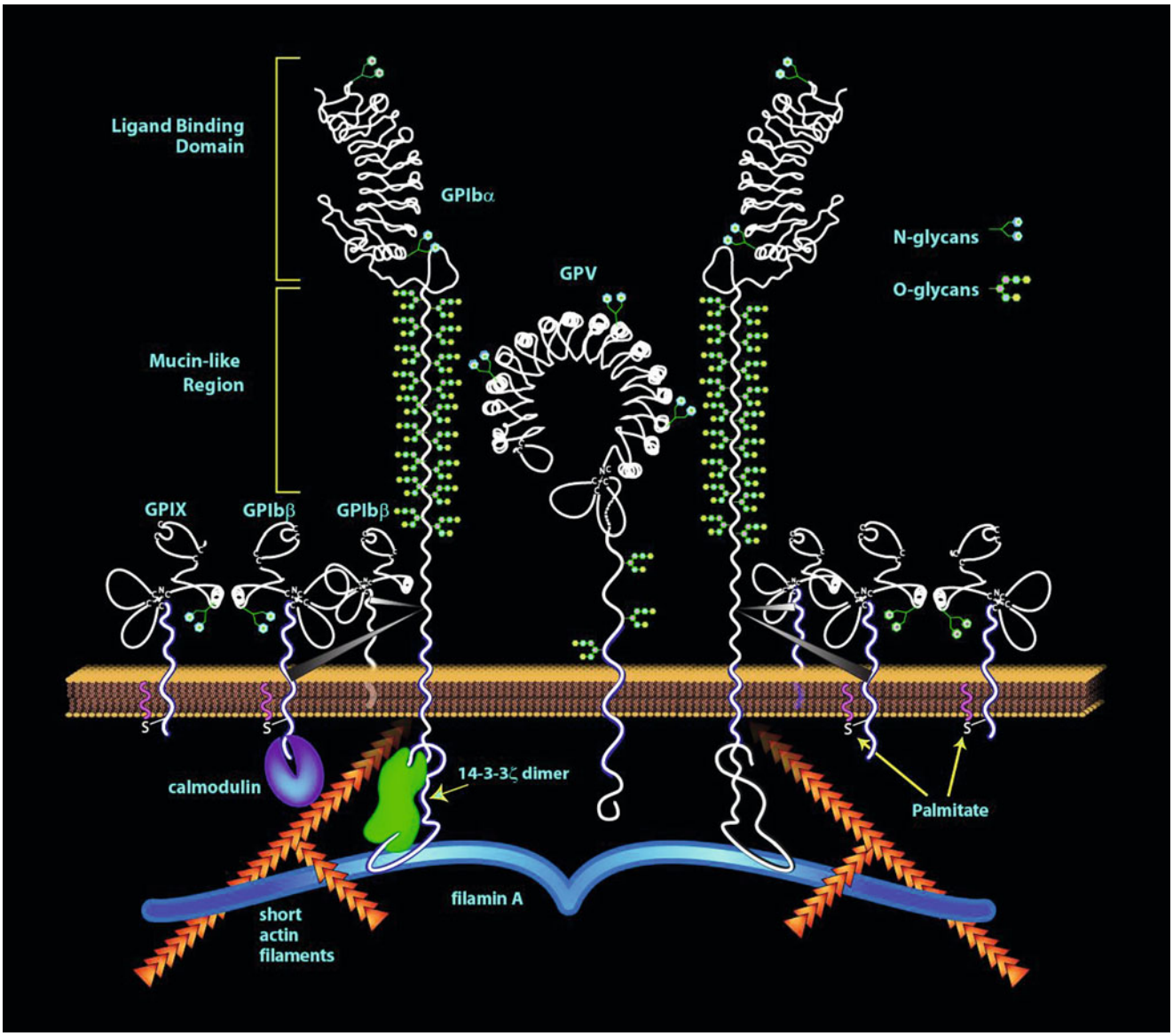

Fig. 1 Schematic representation of the glycoprotein Ib-IX-complex and associated cytoplasmic proteins

phylogenetically widespread leucine-rich repeat protein superfamily (Bella et al. 2008; López 1994). This protein superfamily is characterized by the presence of a single or tandem copies of a 20–30 amino acid motif containing leucine residues at conserved positions and generally adapting a structure known as an α-/β-horseshoe fold, based on the structure of ribonuclease 1, which was the first leucine-rich repeat structure determined (Bella et al. 2008). In this structure, α-helices alternate with β-strands, the β-strands of consecutive repeats residing on the inside of a curved toroid structure to form parallel β-sheets (Bella et al. 2008). It is this β-sheet that makes the leucine-rich repeat proteins so versatile in being able to bind a variety of ligands. Of interest, the GPIb-IX-V leucine-rich repeat structures that have been determined demonstrate very little α-helical structure, but in GPIbα, with tandem repeats, the β-sheet motif is maintained (Uff et al. 2002).

Within the complex, one GPIbα is disulfide linked to two GPIbβ polypeptides (Luo et al. 2007a), and GPIX is noncovalently, and rather tightly, associated with GPIb ($\alpha\beta_2$) (Du et al. 1987; Luo et al. 2007b). GPV is associated more loosely, as it remains associated in the presence of the mild nonionic detergent digitonin, but dissociates in the presence of another nonionic detergent, Nonidet P-40 (Modderman et al. 1992). This hierarchy of association is reflected in the fact that no complex is expressed on the plasma membrane in the absence of GPIbα, GPIbβ, or GPIX, but a GPIb-IX complex can be expressed in the absence of GPV (López et al. 1992a). Whether GPV is fully complexed with GPIb-IX at all times on the platelet

membrane is unknown. Assembly of the complex occurs cotranslationally; in other words, as soon as the polypeptides are synthesized and inserted into the membrane of the endoplasmic reticulum, the four polypeptides assemble into an immature complex (Dong et al. 1998). Recent studies indicate that the primary regions mediating the associations of the GPIb-IX-V polypeptides are the transmembrane domains of the polypeptides (Luo et al. 2007b; Mo et al. 2006, 2012). The GPIbα transmembrane domain associates with two GPIbβ polypeptides, and also with GPV, whereas the GPIX transmembrane domain binds GPIbβ. Full maturation of the complex, as determined by studies in transfected heterologous cells, requires approximately 3 h (Dong et al. 1998). The fully mature complex has a number of posttranslational modifications, including extensive N- and O-glycosylation (López 1994), tyrosine sulfation (Dong et al. 1994), and palmitoylation of Cys residues on the cytoplasmic tails of GPIbβ and GPIX (Muszbek and Laposata 1989).

Polypeptide Structures

Glycoprotein Ibα GPIbα is not only the largest of the GPIb-IX-V polypeptides, it is also the one that binds most of the extracellular ligands and cytoplasmic molecules that interact with the complex. GPIbα is polymorphic in length in the human population, being present in any of four different lengths based on variable numbers of a 13-amino acid sequence present once or repeated up to three additional times within the mucin-like macroglycopeptide region (Ishida et al. 1995; López et al. 1992b). These variants are designated A, B, C, and D, from largest two smallest, the D isoform containing 610 amino acids in its mature form (López et al. 1987). When specific regions or amino acid residues are discussed in the literature, the numbering of the residue positions is usually based on the sequence of the D isoform. This polypeptide has an N-terminal extracellular region of approximately 485 amino acids, a transmembrane domain of 31 amino acids, and a cytoplasmic domain of 94 amino acids (López et al. 1987) (see Fig. 1). The extracellular region can be subdivided into three individual domains. The 300 amino acids at the N-terminus constitute the ligand-binding domain, containing binding sites for all of the known GPIb-IX-V ligands. This domain is characterized by the presence of seven leucine-rich repeats flanked by conserved disulfide loop structures at both the N- and C-termini (López 1994). Following the C-terminal flank is a highly acidic sequence rich in aspartic acid and glutamic acid residues that also contains three tyrosine residues that undergo posttranslational sulfation (Dong et al. 1994; Ward et al. 1996). This anionic sulfated region contains 13 negative charges within a stretch of 19 amino acids, a feature important in determining its ability to bind thrombin exosites I and II (Zarpellon et al. 2011). The ligand-binding domain also contains two N-linked glycan chains, three disulfide bonds, and an exposed, unpaired Cys residue. The crystal structure of this region has been solved, both as an isolated domain (Uff et al. 2002), and in complex with ligands VWF (Dumas et al. 2004; Huizinga et al. 2002) and thrombin (Celikel et al. 2003; Dumas et al. 2003). The GPIbα N-terminus forms a cupped-hand-like structure, with the palm of the hand representing the concave β-sheet surface of the leucine-rich repeats, the fingertips representing the N-terminal disulfide loop known as the β-finger, and the thumb representing the β-switch C-terminal to the leucine-rich repeats (López and Munday 2009).

C-terminal to the anionic sulfated region is a domain known as the macroglycopeptide that is rich in Thr, Ser, and Pro residues. This sequence has 59 potential sites for O-glycosylation and two sites with a consensus for N-glycosylation. Addition of one copy of the repeated sequence adds five additional sites for potential O-glycosylation, the largest (A) form therefore containing up to 15 additional O-glycan chains. The macroglycopeptide is indeed heavily O-glycosylated, carbohydrate in this region accounting for approximately half of the molecular mass of GPIbα as it migrates on SDS-polyacrylamide gels (Okumura et al. 1976). The heavy glycosylation and high Pro content of the macroglycopeptide prevents the region from developing extended secondary structure, the consequence being that the region functions as a test-tube-brush–like spacer to separate the ligand-binding domain from the platelet plasma membrane (see Fig. 1). This feature is important for the normal function of GPIbα as demonstrated by the fact that expression of a complex containing a GPIbα mutant with deletion of the sequence 318–452 (which includes most of the macroglycopeptide) produced a receptor that was expressed normally on the cell surface and could bind VWF under static conditions but which was incapable of mediating the capture of cells to a VWF surface under flow (Li et al. 2002).

It is not clear where the region of glycosylation ends, but the region of high Pro content ends after residue 420. The region 421–483 contains not only fewer Pro residues, it also has fewer Ser and Thr residues that can be O-glycosylated. This region contains what has been termed a "mechanosensitive domain" based on the fact that it has been shown to unfold when tensile force is applied to the extracellular domain after the formation of VWF–GPIbα bonds (Zhang et al. 2015). This unfolding was proposed to be necessary for the transmission of extracellular mechanical signals generated after ligand binding to the N-terminus. Following the mechanosensitive domain are vicinal Cys residues involved in asymmetric disulfide bonds with two GPIbβ peptides (Luo et al. 2007a). The GPIbα transmembrane domain is typical for a type I transmembrane protein,

but its specific sequence is required for interaction with GPIbβ and GPV (Li and Emsley 2013). The ~94 amino acid GPIbα cytoplasmic domain is unique among proteins (no known paralogs) and is notable for what it does and does not contain. It does not contain a domain recognizable as a tyrosine kinase, nor does it contain ITAM sequences. It also only contains one Tyr residue but not within a sequence motif that favors its phosphorylation. This region does, however, contain five sequences that loosely fit consensus sites for glycosylation by Ser/Thr kinases, particularly protein kinase A (PKA). Several sites have been identified as binding sites for the adaptor protein 14-3-3ζ (Andrews et al. 1998; Du et al. 1996; Mangin et al. 2004; Yuan et al. 2009), and these sites have several functions, including regulating the ability of GPIb-IX-V to bind VWF (Yuan et al. 2009), attachment of the complex to the membrane skeletal protein filamin A (Yuan et al. 2009), and downstream signal transduction (Zhang et al. 2012). Interaction of the GPIbα cytoplasmic domain with 14-3-3ζ appears to require phosphorylation of serine residues, particularly at sites Ser609, Ser559, and Ser587/Ser590, but little evidence exists that the state of the phosphorylated residues is dynamic, as it has not been possible to metabolically label GPIbα with radioactive phosphate (Fox et al. 1979; Wardell et al. 1989). Nevertheless, Bodnar et al. and Yuan et al. used sequence-specific anti-phosphopeptide antibodies to identify phosphorylation at Ser609 and 559, respectively (Bodnar et al. 1999; Yuan et al. 2009). Using a phosphoSer609-specific antibody, Bodnar et al. (1999) found that in unstimulated platelets, virtually all GPIbα molecules were phosphorylated at Ser609. These investigators later found that both the unphosphorylated and phosphorylated C-terminal peptides had the capacity to bind 14-3-3ζ, with a phosphorylated peptide being capable of competing with GP Ibα for 14-3-3ζ association while the corresponding non-phosphorylated peptide was not (Bodnar et al. 1999). Binding sites for 14-3-3ζ have also been reported within the sequences Arg557–Gly575 (Mu et al. 2008) and Leu580–Ser 590 (Mangin et al. 2004). The filamin A binding site was first identified to be located between residues 536–568 (Andrews and Fox 1992), later refined to 557–575 (Feng et al. 2003), with residues Phe568 and Trp570 being vital for the interaction (Cranmer et al. 2005). A crystal structure of a complex between the GPIbα peptide 556–577 and filamin A domain 17 reveals that this region of GPIbα contributes a β-strand to a bimolecular β-sheet (Nakamura et al. 2006). In addition to binding 14-3-3ζ and filamin A, the GPIbα contains a binding site for phosphoinositide (PI)-3 kinase (Mu et al. 2008; Munday et al. 2000).

Glycoprotein Ibβ and Glycoprotein IX GPIbβ and GPIX are both much smaller polypeptides than GPIbα, their mature forms being of lengths 181 and 161 residues, respectively. These two polypeptides have closely related extracellular structures, with approximately 60 % sequence similarity, but their cytoplasmic sequences are completely divergent (López 1994). The extracellular domains each essentially comprise one leucine-rich repeat domain containing one copy each of the leucine-rich sequence that characterizes these domains and conserved N- and C-terminal flanking sequences. The flanking sequences form two disulfide bonds N-terminal to the leucine-rich repeat and two disulfide bonds C-terminal to the domain. All Cys residues present in GPIbβ are conserved in GPIX except one, which lies immediately before the transmembrane domain in GPIbβ and is replaced by Ser in GPIX. This GPIbβ Cys forms a disulfide bond with either Cys484 or Cys485 of GPIbα, the mature complex containing two GPIbβ subunits asymmetrically disulfide bonded to one GPIbα (Luo et al. 2007a). GPIbβ and GPIX can form a complex independently of the other polypeptides (López et al. 1994), the binding sites between the two involving the transmembrane and extracellular domains (Li and Emsley 2013). The GPIbβ transmembrane domain also binds directly to the GPIbα transmembrane domain (Luo and Li 2008). The cytoplasmic domains of GPIbβ and GPIX are both relatively short, that of GPIX containing only seven residues and that of GPIbβ containing approximately 34. Both contain Cys residues in the immediate juxtamembrane position of the cytoplasmic domain which have been shown to become posttranslationally palmitoylated (Muszbek and Laposata 1989), a modification that may help to localize the complex to membrane microdomains, although evidence exists that this modification is not required for raft association (Xu et al. 2015). No other functions have been described for the short GPIX cytoplasmic domain, but the GPIbβ sequence has at least two important features of functional importance. Immediately following the palmitoylated Cys residue is an 18-residue sequence that contains eight Arg residues and ends with a Ser (Ser166) known to be phosphorylated by PKA (Wardell et al. 1989). The membrane-proximal Arg-rich sequence can bind both calmodulin and tumor necrosis factor receptor-associated factor 4 (TRAF4) (Andrews et al. 2001; Arthur et al. 2011). Calmodulin binding in this region has been proposed to regulate access to a cleavage site on GPIbα for membrane metalloproteinases of the ADAM family (Aktas et al. 2005; Gardiner et al. 2007), because treatment with the calmodulin inhibitor W7 induces GPIbα cleavage. Nevertheless, the relationship between GPIbβ calmodulin binding and GPIbα proteolysis is not direct, as mutation of two GPIbβ residues prevented calmodulin binding but had no effect on GPIbα shedding (Mo et al. 2010). TRAF4 association with GPIbβ and with GPVI is proposed to link ligand binding to GPIbα to downstream redox pathways involving platelet NADPH oxidase (Arthur et al. 2011). At the C-terminus of this Arg-rich basic sequence, Ser166 becomes phosphorylated when the

cytosolic cAMP concentration rises, thereby mediating one of the platelet inhibitory effects of substances such as prostaglandins I_2 and E_1 (Wardell et al. 1989). Phosphorylation of Ser166 provides another binding site within the complex for 14-3-3ζ (Andrews et al. 1998; Calverley et al. 1998), which likely mediates the platelet inhibitory actions.

Glycoprotein V GPV is the second largest polypeptide of the complex but so far the most mysterious and least studied. GPV was the first identified target for thrombin proteolysis on the platelet plasma membrane, cleavage producing a soluble fragment, GPVf1, that circulates in the plasma (Mosher et al. 1979; Phillips and Agin 1977). The concentration of GPVf1 in normal plasma is approximately 17 ng/mL based on measurements from 100 normal individuals (Azorsa et al. 1999). This concentration increases during thrombotic episodes (Morel et al. 2004; Ravanat et al. 2000).

GPV contains 544 amino acids, of which 504 are extracellular, 22 comprise a transmembrane domain, and only 18 reside within the cytoplasm (Lanza et al. 1993). The extracellular region is heavily glycosylated (Berndt and Phillips 1981) and contains a structure similar to that of the other GPIb-IX-V complex polypeptides, with 15 leucine-rich repeats flanked by conserved N- and C-terminal disulfide loops. A thrombin cleavage site, after Arg460, is situated only a few residues from the end of the C-terminal flank. It is of interest to note that the thrombin cleavage site is conserved in humans, mice, and rats (Ravanat et al. 1997). The GPV cytoplasmic domain contains only 18 amino acids, but this sequence has been shown to bind calmodulin (Andrews et al. 2001). As with other platelet receptors, inhibition of calmodulin binding to the GPV cytoplasmic domain also induces GPV shedding, a process that has been shown to be mediated by ADAM17 (Gardiner et al. 2007).

The most important physiological function of GPV is still very likely undetermined. From studies of deficient mice, the function of this protein is not obvious. GPV is not necessary for expression of the GPIb-IX-V complex, although its own expression is enhanced in the presence of the other subunits (Kahn et al. 1999; Li et al. 1995). GPV-deficient platelets are morphologically normal. In addition, GPV-deficient mice have been shown to lack a hemostatic defect (Kahn et al. 1999) and to have normal thrombin signaling. In contrast, however, other studies showed GPV-deficient platelets to be more responsive to thrombin (Ramakrishnan et al. 2001), and studies in thrombosis models demonstrated increased platelet adhesion and aggregation that was dependent on both thrombin and VWF (Ni et al. 2001). More recent studies of deficient mice backcrossed into the C57B/6 background indicate that the effect on thrombus size and survival after agonist injection depends on the nature of the injury and the agonist injected (Nonne et al. 2008).

Functions of the Complex

The GPIb-IX-V complex has many physiological ligands, which include adhesion molecules such as VWF, thrombospondin-1, and fibrin, membrane counter-receptors on leukocytes ($\alpha_M\beta_2$) and endothelial cells (P-selectin), and a variety of plasma proteins, including thrombin, factors XI and XII, and high molecular weight kininogen (López et al. 1998). In addition, the complex has been shown to recognize misfolded proteins with amyloid properties, triggering platelet activation (Herczenik et al. 2007). Here, we will limit the discussion to the interactions of the complex with VWF, thrombin, and $\alpha_M\beta_2$.

von Willebrand Factor Interaction VWF is an enormous plasma protein produced in endothelial cells (the major plasma source) and megakaryocytes and stored in endothelial Weibel–Palade bodies and platelet α-granules. In healthy individuals, the normal VWF concentration in the plasma is approximately 10 μg/mL but can be much higher in disease states, particularly those characterized by systemic endothelial activation (Schwameis et al. 2015). Given that platelets and VWF both circulate, a mechanism must exist to prevent VWF from binding GPIb-IX-V before there is a hemostatic need. Indeed, under static conditions with no flow, the interaction between the two molecules requires the presence of a modulator, usually the antibiotic ristocetin from the soil bacterium *Nocardia lurida* (Howard and Firkin 1971), or botrocetin, a C-type lectin from the venom of the pit viper *Bothrops jararaca* (Andrews et al. 1989; Read et al. 1989). Ristocetin binds VWF in a region next to the GPIbα-binding A1 domain (Azuma et al. 1993; Berndt et al. 1992; Girma et al. 1990) and changes VWF's conformation to one that favors GPIbα binding, whereas botrocetin promotes the formation of a trimolecular complex (Fukuda et al. 2005). In vivo, the binding of VWF to collagen at the site of vascular injury immobilizes VWF in a conformation that can bind platelets. In addition, elevated shear stress can alter the conformation of VWF to enable it to bind GPIb-IX-V even in the fluid phase (Chen and López 2005b). The two molecules also interact on the surface of intact endothelium in the setting of deficiency of the VWF-cleaving metalloproteinase ADAMTS13 (Dong et al. 2002), a situation that leads to the devastating microvascular thrombotic disorder, thrombotic thrombocytopenic purpura.

Shear stress, in addition to exposing GPIbα-binding sites on VWF, also influences the GPIbα–VWF bond by exerting force on the bond. The bond is regarded as a catch bond: a bond that increases in lifetime when force is applied to it (Liu et al. 2015; Thomas 2009). A consequence of this bond characteristic is that the attachment of platelets to a VWF surface becomes tighter as shear stress increases, explaining the importance of the VWF-GPIbα bond in regions of the

vasculature with the highest shear, where platelets accumulate very efficiently (Weiss et al. 1978), both because of increased bond lifetime and enhanced delivery of platelets. Recently, evidence has emerged that increased bond lifetimes can also result from tension exerted on the bond from the inside of the platelet through cytoskeletal forces after the platelets have adhered (Feghhi et al. 2016). In this way, this adhesive complex behaves like the platelet integrin $\alpha_{IIb}\beta_3$: it is capable of both inside–out and outside–in signaling. But, in contrast to the integrin, the sequence of these signaling processes is reversed. In the interaction of GPIb-IX-V with VWF, the complex first signals *into* the platelet after engaging VWF, and these signals then activate cytoskeletal machinery to pull on the GPIbα cytoplasmic tail to reinforce the bond with VWF on the *outside* of the platelet.

Crystal structures of GPIbα–VWF A1 reveal that GPIbα interacts with the VWF A1 domain through the concave side of the leucine-rich repeat motif, but the interacting surface is discontinuous, primarily involving sequences from the N- and C-terminal flanking disulfide loops. At both the N- and C-termini, the GPIbα interaction sites involve β-hairpin structures, known as the β-finger and β-switch, respectively. The β-switch C-terminal loop goes from a disordered structure to a β-hairpin upon A1 domain binding, contributing two β-strands to an eight-stranded intermolecular β-sheet that stabilizes the interaction (Dumas et al. 2004; Huizinga et al. 2002) (see Fig. 2). Within the leucine-rich repeat region, leucine-rich repeats 5–8 contribute to the interaction, the remainder of the leucine-rich repeats making only long-range electrostatic contacts with the A1 domain. Recently, evidence has been presented that when force is applied to the GPIbα–A1 bond, the GPIbα N-terminus unfolds to some extent, which could account for or contribute to the catch bond nature of the interaction (Ju et al. 2016).

Role of Lipid Rafts Although all of the sequences necessary for GPIbα to bind VWF appear to be present within the N-terminal 300 amino acids of GPIbα and attachment of a polypeptide bearing this sequence is sufficient to allow coated beads to bind VWF, the binding of platelets to VWF requires that the GPIb-IX-V complex be positioned appropriately within the platelet plasma membrane. Early studies in transfected heterologous cells revealed that truncation of as few as six amino acid residues from the GPIbα C-terminus increased diffusion of the complex within the plane of the plasma membrane and diminished the ability of the cells to bind VWF in the presence of ristocetin (Dong et al. 1997). Subsequent studies by several groups suggested that this effect is the result of disrupting the binding of 14-3-3ζ and filamin A to the GPIbα cytoplasmic domain (Feng et al. 2000, 2003; Williamson et al. 2002).

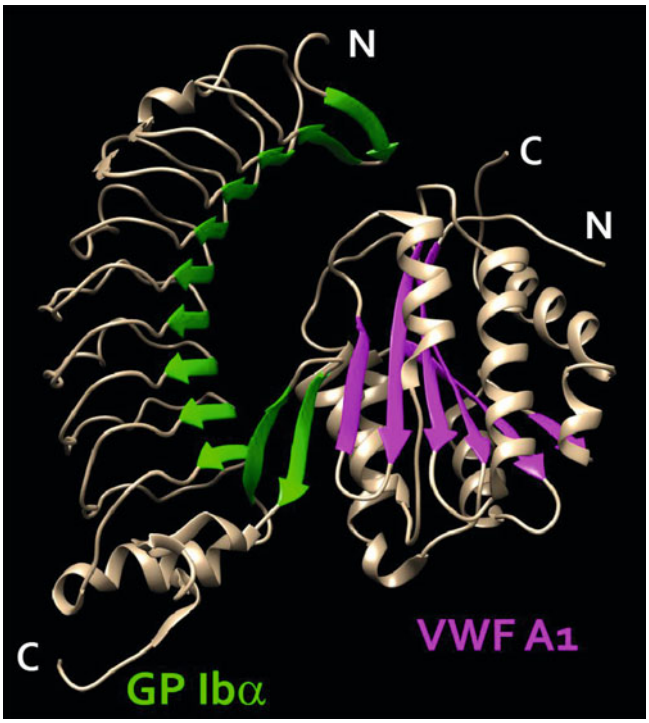

Fig. 2 Crystal structure of the GPIbα N-terminus bound to the VWF A1 domain. *Green* shows GPIbα β-strands from the N-terminal β-finger, through the leucine-rich repeats, to the C-terminal β-switch. Note that the β-switch contributes to β-strands to an 8-strand β-sheet between GPIbα and VWF. The VWF β-sheet is shown in *magenta*. Structures based on structures from reference (Huizinga et al. 2002)

Another important requirement for the VWF-binding function of the complex is localization of the complex to lipid raft domains, regions of the plasma membrane that represent dynamic assemblies of sphingolipids and cholesterol with greater order than the surrounding phospholipid-rich regions of the membrane (López et al. 2005). In unactivated platelets, only a small percentage of all GPIb-IX-V complexes are present in the lipid raft fraction. The quantity of the complex associated with rafts increases three- to sixfold when platelets are activated by VWF but not when activated by ADP (Shrimpton et al. 2002). Raft localization of the complex is required for platelets to be able to adhere to VWF surfaces, although the mechanism for this is not understood. One possibility is that raft localization requires, or is required for, the complex to associate with the platelet membrane skeleton (Munday et al. 2010), an association that when disrupted also disrupts the ability of platelets to bind VWF (Feng et al. 2000; Williamson et al. 2002). Another very likely consequence of complex raft localization is the need to effectively transmit downstream signals after the complex engages VWF. Raft regions are known to attract, on their cytoplasmic sides, signaling molecules such

as kinases and adaptor proteins, particularly if they are modified by cysteine acylation (López et al. 2005). This feature enables signal transduction. For example, it was shown that after VWF binds to GPIb-IX-V, only the populations of phospholipase Cγ2 and the tyrosine kinase Syk that reside in lipid rafts become phosphorylated (Jin et al. 2007). Raft localization of the complex also allows the complex to contact other molecules, with functional consequences. For example, GPIb-IX-V and the platelet Fc receptor, FcγRIIa, physically associate (Sullam et al. 1998), but only within lipid rafts (Shrimpton et al. 2002). Raft association is important for FcγRIIa to be able to transmit intracellular signals, a requirement that explains why some GPIbα antibodies are able to inhibit activation of platelets by immune complexes or aggregated IgG (Sullam et al. 1998).

Congenital Hemorrhagic Disorders Affecting the GPIb-IX-V Complex The interaction between the GPIb-IX-V complex and VWF can be disrupted by naturally occurring mutations. When those mutations affect GPIbα, they produce Bernard–Soulier syndrome (BSS) or platelet-type (pseudo) von Willebrand disease (VWD), and when they affect VWF, they produce VWD. Mutations in GPIbα, GPIX, or GPIbβ can cause BSS, usually producing unstable proteins that cannot be incorporated into a complex before they are removed by the cell's quality control system (Andrews and Berndt 2013; López et al. 1998). A small number of mutations of GPIbα have been described that yield mutant complexes defective in VWF binding (Savoia et al. 2014).

The prevalence of BSS is very low, probably less than one per 1,000,000 in the population. Patients with the full-blown disorder usually present in early childhood, and typically have findings of mucocutaneous bleeding: epistaxis, gingival and cutaneous bleeding, and menorrhagia in females (López et al. 1998). Laboratory findings include thrombocytopenia, extremely large platelets on the blood smear, and defective ristocetin-induced platelet agglutination with normal ristocetin cofactor activity. Almost all BSS mutations that have been described are autosomal recessive, requiring the presence of two mutant alleles. One exception has been described in a family in which BSS is caused by a single allele mutation that converts Asn41 of GPIbα to His (Vettore et al. 2008). Patients with this mutation appear to have a mild form of the syndrome, and normal levels of GPIbα on their platelets, suggesting a dominant-negative mode of inheritance. Recently, an Indian family was described in which only one GPIbα allele was found to be mutated, a missense mutation that substituted GPIbα Val31 with Leu (Ali et al. 2016). Whether this represents a dominant-negative mutation cannot be determined from the characterization of the patient's proteins. Although dominant transmission is unusual, heterozygosity for recessive mutations is not necessarily without phenotype, as heterozygosity for a common BSS allele (Ala156Val) has been shown to be responsible for most of the cases of macrothrombocytopenia seen in Italy (Noris et al. 2012). With this mutation, thrombocytopenia is usually mild; in one study only 18 of 103 patients examined had platelet counts below 50,000/μL (Noris et al. 2012).

Selected naturally occurring mutations of GPIbα also produce a polypeptide that can bind VWF in the absence of shear stress or modulators, a condition known as platelet-type VWD (Othman et al. 2011). Four naturally occurring mutations have been described in the GPIbα β-switch region: M239V, G233V, G233S, and D235Y. These mutations produce structural changes that favor the conversion of the β-switch region from a disordered loop to a β-hairpin as is found in the GPIbα–VWF complex (Othman et al. 2011). Another very interesting mutation that produces platelet-type VWD involves an in-frame deletion of 27 nucleotides encoding a stretch of nine amino acids (del420–428) (Chen and López 2005a; Othman et al. 2005). This mutation involves a sequence within the recently identified "mechanosensitive domain" (Zhang et al. 2015), but how deletion of this sequence induces spontaneous VWF binding is still a mystery. Platelet-type VWD mutations, although gain of function, produce a bleeding phenotype because they induce clearance of the largest and hemostatically most active VWF multimers from the circulation. An analogous condition is produced by mutation of VWF: type 2B VWD. The fact that gain-of-function mutations of both GPIbα and VWF can induce the two molecules to interact spontaneously indicates that the mechanism by which shear stress regulates the bond is not merely by revealing a previously cryptic A1 domain in VWF. If this were true, there would be no way in which mutations in GPIbα would be able to induce exposure of the A1 domain. Thus, binding accessibility appears to be regulated at the level of both the receptor and the ligand.

Role of the GPIb-IX-V Complex in the Actions of Thrombin The role of the GPIb-IX-V complex as a major thrombin-binding site on platelets has been appreciated for over four decades (López 1994). Studies of equilibrium thrombin binding to the platelet membrane identified both high-affinity and moderate-affinity sites, present at 500–4000 and 20,000–50,000 per platelet, respectively (Harmon and Jamieson 1986; Shuman and Majerus 1975; Workman et al. 1977). From studies of BSS platelets, it was clear that both the high- and moderate-affinity sites reside with the GPIb-IX-V complex (Jamieson and Okumura 1978), and Okumura et al. (1978) determined that the 45-kDa N-terminal fragment of GPIbα contained both sites. Binding of thrombin to the GPIb-IX-V complex is necessary for platelet activation at low concentrations of

the agonist (Jamieson and Okumura 1978), an activation that also involves proteolysis of the G-protein-coupled receptor PAR-1 (Vu et al. 1991). The cleavage of PAR-1 is accelerated by binding of thrombin to GPIb-IX-V (De Candia et al. 2001), and removal or absence of GPV yields a signaling function to the complex upon thrombin binding that no longer requires thrombin's protease activity (Ramakrishnan et al. 2001). Signals generated after thrombin binds GPIb-IX-V activate the 14-3-3/Rac-1/LIMK-1 pathway that is also involved in transmitting signals after GPIb-IX-V engages VWF (Estevez et al. 2016).

Thrombin binds exclusively within the N-terminal 300 amino acids of GPIbα. Two structures of this region in complex with thrombin have been determined (Celikel et al. 2003; Dumas et al. 2003). Both structures show two thrombin molecules bound to one GPIbα N-terminus, and both of the thrombin interacting sites reside in the GPIbα ligand-binding domain, involving sequences in the C-terminal flanking region and the anionic sulfated region. Nevertheless, the two structures are in fact different, the difference involving flexibility of the anionic sulfated tail (Sadler 2003). The two molecules of thrombin bind to GPIbα through distinct regions raising the possibility that each thrombin can also bind two GPIbα molecules. Figure 3 depicts a bimolecular complex based on a crystal structure of the complex from Celikel et al. (Celikel et al. 2003) in which thrombin interacts with GPIbα at two sites: in the C-terminal flank of GPIbα and in the anion sulfated region. This binding is compatible with data from monoclonal antibody blocking studies. Antibodies that map to the C-terminal flank (TM60 (Yamamoto et al. 1985) and VM16d (Mazurov et al. 1991)) or to the anionic sulfated region (SZ2 (Ward et al. 1996) and LJIb10 (Mazzucato et al. 1998)) are all capable of blocking thrombin binding to GPIbα (Shen et al. 2000). AP1 is another antibody that blocks low-dose thrombin aggregation (Pidard et al. 1982), and its epitope has been mapped in detail within the sequence Glu212–Tyr228 (Peng et al. 2004). Thus, at low thrombin concentrations, thrombin may form a multimolecular complex with GPIb-IX-V in which each molecule of GPIbα binds two thrombin molecules, and each thrombin in turn is bound to two GPIbα molecules. The minimal size of a complex that would allow each bound thrombin to bind two GPIbα polypeptides would contain four GPIbα and four thrombin molecules and would resemble a ring structure.

The capacity of one GPIbα to bind two molecules of thrombin also raises the possibility that once the first thrombin is bound, the second binds more readily, yielding a cooperative interaction. Alternatively, the binding of the second thrombin could exhibit negative cooperativity. In fact, early studies from Tollefsen and Majerus (1976) of thrombin binding to platelets demonstrated that both high- and low-affinity sites resided within the same molecule and thrombin binding exhibited negative cooperativity.

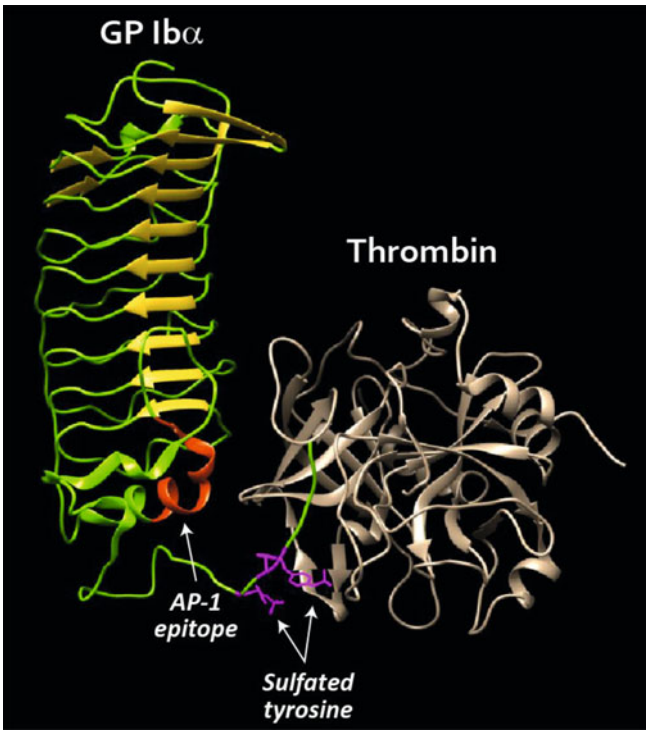

Fig. 3 Crystal structure of a complex between one GPIbα N-terminus and one thrombin molecule. Note that thrombin makes two major contacts with GPIbα, with the α-helical region at the C-terminal flank of the GPIbα ligand-binding domain that contains the epitope for the monoclonal antibody AP1 (shown in *orange*) and with the anionic sulfated region. The sulfated tyrosines are shown in *magenta*. Based on one of the complexes from reference (Celikel et al. 2003)

The Interaction of the GPIb-IX-V Complex with Leukocyte $\alpha_M\beta_2$ (MAC-1) Another important counter-receptor for the platelet GPIb-IX-V complex is the leukocyte integrin $\alpha_M\beta_2$ (Simon et al. 2000). Search for a counter-receptor on platelets for leukocyte $\alpha_M\beta_2$ was inspired by the observation that experimental arterial restenosis in mice after vessel injury could largely be prevented by depletion of either neutrophils or platelets or by the absence of $\alpha_M\beta_2$ (Simon et al. 2000). One characteristic of $\alpha_M\beta_2$ that provided a clue to the identity of the counter-receptor was the presence in the α_M chain of an inserted domain (I-domain). I-domains are found in many integrins and are usually involved in adhesion. They also bear a striking structural similarity to the A domains of VWF, including the GPIbα-binding A1 domain. Examination of the potential for GPIbα to serve as a platelet counter-receptor for $\alpha_M\beta_2$ showed that the two membrane proteins could indeed interact, the interaction involving the N-terminal 45-kDa ligand-binding domain of GPIbα and

the α_M I-domain (Simon et al. 2000). Within the α_M I-domain, the region was localized to a short sequence, Pro201 to Lys217 (Ehlers et al. 2003). The I-domain from the related integrin $\alpha_L\beta_2$ does not interact with GPIbα, but when the α_L I-domain was mutated so as to replace only two amino acids with the corresponding $\alpha_M\beta_2$ residues from the GPIbα-binding site, α_L gained the capacity to bind GPIbα (Ehlers et al. 2003). Within GPIbα, binding to $\alpha_M\beta_2$ involves sequences C-terminal to the leucine-rich repeat regions, as the binding is blocked by two antibodies that bind within this region AP-1 and VM16d (Simon et al. 2000). Of these two antibodies, only AP-1 interferes with VWF binding, indicating that the binding sites within GPIbα are distinct. A crystal structure of the complex has yet to be reported.

In addition to its role in restenosis after vessel injury, the GPIbα–$\alpha_M\beta_2$ interaction is important in other inflammatory conditions. In a model of experimental thrombotic glomerulonephritis induced by lipopolysaccharide and anti-glomerular basement membrane antibodies, the severity of glomerular thrombosis was attenuated by neutrophil depletion or by treatment with an antibody that targets the GPIbα-binding region of the α_M I-domain, an antibody known as anti-M2 (Hirahashi et al. 2009). Likewise, the GPIbα–$\alpha_M\beta_2$ interaction is very likely to play a role in multiple sclerosis (MS). Platelets have been shown to localize to the demyelination plaques of the brain and spinal cord in both humans with MS and mice with experimental autoimmune encephalomyelitis (EAE), the mouse disease that models human MS (Langer et al. 2012). Platelet depletion significantly improved the clinical disease scores and diminished the CNS inflammatory response in EAE (Langer et al. 2012). A similar amelioration of CNS inflammation was observed in mice treated with a rat monoclonal antibody against mouse GPIbα or with anti-M2. These two examples illustrate the importance of the GPIbα–$\alpha_M\beta_2$ interaction in inflammatory conditions. This interaction therefore represents a therapeutic target for anti-inflammatory drug development. Further, the fact that the binding of $\alpha_M\beta_2$ to GPIbα can be selectively targeted by GPIbα inhibitors indicates that platelets can be targeted in inflammatory disorders without compromising their hemostatic function.

GPIb-IX-V Complex Signaling

Signals are transmitted across the platelet plasma membrane by the engagement of the GPIb-IX-V complex by either VWF or thrombin and probably by other interactions. These signals activate integrins $\alpha_{IIb}\beta_3$ (Kasirer-Friede et al. 2004) and $\alpha_2\beta_1$ (Cruz et al. 2005) and thereby enable stable platelet adhesion and subsequent aggregation. As mentioned above, localization of the complex to lipid raft domains is a requirement for this signal transduction (Jin et al. 2007; Shrimpton et al. 2002), bringing the complex in proximity to a variety of signaling molecules. Attached to the cytoplasmic domains of the GPIb-IX-V polypeptides are a variety of molecules involved in signal transduction, including 14-3-3ζ, PI3-kinase, filamin, calmodulin, and Src family kinases (Canobbio et al. 2004). In addition, the GPIb-IX-V complex associates with two other membrane proteins capable of signal transduction: GPVI/Fcγ chain complex (Wu et al. 2001) and the platelet low-affinity Fc receptor, FcγRIIa (Sullam et al. 1998). Both of these receptors contain cytoplasmic motifs known as immunoreceptor tyrosine-based activation motifs (ITAMs) which, when phosphorylated by Src family tyrosine kinases, attract the tyrosine kinase Syk, which phosphorylates downstream effectors. GPIb-IX-V associates with these receptors primarily in lipid raft domains (Ozaki et al. 2013; Shrimpton et al. 2002).

Association of GPIbα with both filamin (Feng et al. 2003) and 14-3-3ζ (Dai et al. 2005) is required for binding VWF, as is localization to lipid rafts (Shrimpton et al. 2002). Upon engagement of the complex by VWF, PI-3 kinase activates the downstream Ser/Thr kinase Akt, and FcγRIIa (Canobbio et al. 2001) and the GPVI/FcγR chain complex (Falati et al. 1999; Gardiner et al. 2010) become rapidly tyrosine phosphorylated by Src family tyrosine kinases. This phosphorylation induces recruitment of Syk to the ITAMs and its subsequent activation with downstream phosphorylation of phospholipase (PL) Cγ2 and LAT (Wu et al. 2001). Wu et al. (2001) observed that despite the fact that VWF/botrocetin stimulated phosphorylation of PLCγ2, the stimulus did not lead to the production of inositol 1,4,5 trisphosphate (IP_3) or an elevation in cytosolic Ca2+ concentration. Other investigators, however, have reported calcium fluxes after GPIbα engagement by immobilized VWF (Kasirer-Friede et al. 2004; Mazzucato et al. 2002) or during shear-induced platelet aggregation (Chow et al. 1992).

The binding of VWF to the GPIb-IX-V complex is inhibited in at least two ways: by GPIbβ phosphorylation (Bodnar et al. 2002) and PECAM-1 engagement (Jones et al. 2014). GPIbβ is phosphorylated by PKA when cAMP concentrations rise and requires localization of the complex to rafts, along with the A-kinase anchoring protein moesin (Raslan et al. 2015). PECAM-1 induces the internalization of the GPIb-IX-V complex through a pathway that involves AKT and glycogen synthase kinase and requires the involvement of the cytoskeleton and the $\alpha_{IIb}\beta_3$ integrin.

Summary

The GPIb-IX-V complex is a remarkable platelet membrane receptor that carries out many functions important for normal physiology. Despite the fact that it has been studied

extensively for over half a century, much remains to be learned about its structure, functions, membrane topology, signaling functions, and involvement in diseases. These aspects will remain fertile fields of investigation.

Take-Home Messages

- The GPIb-IX-V complex has many functions in hemostasis, thrombosis, and inflammation.
- Normal expression of the complex requires GPIbα, GPIbβ, and GPIX but not GPV.
- The complex is unique in that no other complexes with a similar organization are found in nature.

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$\alpha_{IIb}\beta_3$ (GPIIb/IIIa) Structure and Function

Joel S. Bennett

Abstract

The platelet membrane protein $\alpha_{IIb}\beta_3$, otherwise known as glycoprotein IIb/IIIa (GPIIb/IIIa), is a member of the ubiquitous integrin family of transmembrane (TM) heterodimers and is absolutely required for platelet aggregation. $\alpha_{IIb}\beta_3$ mediates platelet aggregation when fibrinogen or von Willebrand factor (vWF) bound to active $\alpha_{IIb}\beta_3$ cross-links adjacent stimulated platelets into stable aggregates. $\alpha_{IIb}\beta_3$ heterodimers are assembled from α_{IIb} and β_3 monomers in the endoplasmic reticulum. Mutations impairing the synthesis of either subunit decrease $\alpha_{IIb}\beta_3$ expression, thereby causing the autosomal bleeding disorder Glanzmann thrombasthenia. Conversely, $\alpha_{IIb}\beta_3$-mediated platelet aggregation is responsible for the arterial thrombi that cause heart attack and stroke. $\alpha_{IIb}\beta_3$ is present on platelets in an equilibrium between an inactive bent conformation and an active extended conformation. To prevent spontaneous platelet aggregation, however, $\alpha_{IIb}\beta_3$ on circulating platelets is constrained to its inactive conformation via intramolecular interactions involving its transmembrane and cytoplasmic domains. When platelets encounter a damaged blood vessel, stimulation by agonists such as thrombin and ADP causes nearly instantaneous $\alpha_{IIb}\beta_3$ activation by inducing talin-1 and kindlin-3 binding to the β3 cytoplasmic domain, thereby relieving the transmembrane and cytoplasmic domain restraints. $\alpha_{IIb}\beta_3$ binds to fibrin as well as fibrinogen. Thus, following $\alpha_{IIb}\beta_3$ activation, $\alpha_{IIb}\beta_3$ bound to fibrin transmits the retraction forces that consolidate fibrin clots, enabling them to resist the potentially disruptive shear forces present in circulating blood.

Introduction

Blood platelets aggregate to form shear-resistant thrombi at sites of vascular injury, thereby providing primary hemostasis when the injury occurs in the microcirculation but also producing vascular obstruction when the injury occurs in coronary and cerebral arteries. Platelet thrombus formation is a highly orchestrated process in which a layer of platelets adherent to the site of injury becomes a nidus for the subsequent aggregation of stimulated circulating platelets. Both platelet adherence and aggregation require the participation of members of the integrin family of adhesion receptors. Integrins are ubiquitous transmembrane (TM) α/β heterodimers that mediate diverse processes requiring cell-matrix and cell-cell interactions such as cellular migration during embryogenesis, cellular adhesion, cancer metastases, and T lymphocyte function (Hynes 2002). In mammals, 18 integrin α subunits and 8 integrin β subunits have been identified. These combine to form 24 different integrin heterodimers. The heterodimers can be grouped into subfamilies according to the identity of the β subunit (Hynes 2002). Platelets express three members of the β_1 subfamily ($\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) mediating platelet adhesion to the extracellular matrix proteins, collagen, fibronectin, and laminins, respectively (Piotrowicz et al. 1988; Ill et al. 1984;

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Sonnenberg et al. 1988; Staatz et al. 1989), and both members of the β_3 subfamily ($\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$). In vitro, $\alpha_v\beta_3$ mediates platelet adhesion to vitronectin and osteopontin (Bennett et al. 1997; Paul et al. 2003), but whether this is important for platelet function in vivo is unclear. By contrast, $\alpha_{IIb}\beta_3$, also known as glycoprotein IIb/IIIa (GPIIb/IIIa), is a receptor for fibrinogen, von Willebrand factor (VWF), fibronectin, and vitronectin on activated platelets and is absolutely required for platelet aggregation in vitro and in vivo (Fig. 1). Moreover, inherited decreases in the level of $\alpha_{IIb}\beta_3$ expression or in $\alpha_{IIb}\beta_3$ function produce the bleeding disorder Glanzmann thrombasthenia (George et al. 1990). Further, $\alpha_{IIb}\beta_3$-mediated platelet aggregation is responsible for the thrombi that form on damaged arteries (Lefkovits et al. 1995). Thus, $\alpha_{IIb}\beta_3$ is indispensable for normal hemostasis and plays a critical role in arterial thrombosis. Consequently, it has been intensively studied and it serves as the paradigm for regulated integrin function. This chapter will review the current knowledge of $\alpha_{IIb}\beta_3$ structure and function.

$\alpha_{IIb}\beta_3$ Biosynthesis

$\alpha_{IIb}\beta_3$ expression is restricted primarily to cells of the megakaryocyte lineage, although α_{IIb} has been detected on embryonic (Dumon et al. 2012) and myeloid-biased adult hematopoietic stem cells (Gekas and Graf 2013) and functional $\alpha_{IIb}\beta_3$ is expressed on mast cells where it participates in chronic inflammation and innate immunity (Oki et al. 2009). $\alpha_{IIb}\beta_3$ is a calcium-dependent heterodimer that dissociates into α_{IIb} and β_3 monomers in the presence of calcium chelators such as EDTA (Jennings and Phillips 1982). α_{IIb} has an unreduced molecular weight of 136,000 and dissociates into a 125,000 molecular weight heavy chain ($\alpha_{IIb}\alpha$) and 23,000 molecular weight light chain ($\alpha_{IIb}\beta$) following disulfide bond reduction. β_3 is a single chain protein containing 56 cysteine residues and 28 disulfide bonds (Fitzgerald et al. 1987); its apparent molecular weight of 90,000 on unreduced SDS gels increases to 110,000 following disulfide bond reduction (Jennings and Phillips 1982). In megakaryocytes, $\alpha_{IIb}\beta_3$ is assembled from α_{IIb} and β_3 precursors in the rough endoplasmic reticulum (ER) (Duperray et al. 1987) after which the assembled heterodimers are transported to the Golgi complex where pro-α_{IIb} undergoes cleavage into heavy and light chains, presumably by furin or a furin-like protease (Kolodziej et al. 1991a). Functional consequences of α_{IIb} cleavage, if any, have not been detected. In the absence of heterodimer formation, monomeric α_{IIb} and β_3 are retained in the ER and are eventually degraded (Kolodziej et al. 1991b). Moreover, as shown by the deleterious effect on $\alpha_{IIb}\beta_3$ expression of point mutations causing Glanzmann thrombasthenia, assembly of $\alpha_{IIb}\beta_3$ heterodimers alone is insufficient to guarantee $\alpha_{IIb}\beta_3$ egress from the ER and misfolded heterodimers are retained as well (Kolodziej et al. 1991b; Poncz et al. 1994). There are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ on the surface of unstimulated platelets (Wagner et al. 1996) and additional copies located in platelet granule membranes are translocated to the platelet surface during platelet secretion (Niiya et al. 1987). The platelets of obligate Glanzmann thrombasthenia heterozygotes aggregate normally, indicating that 50 % of the normal amount of $\alpha_{IIb}\beta_3$ is at least sufficient to support platelet aggregation (McEver JCI 1980). However, the observation that as little as 5 % of the normal amount of kindlin 3, a β_3 binding protein that is required to convert inactive $\alpha_{IIb}\beta_3$ to its active state, is sufficient to prevent spontaneous bleeding in mice suggests that only small amounts of active $\alpha_{IIb}\beta_3$ are required to support basal hemostasis (Klapproth et al. 2015).

The α_{IIb} and β_3 genes, *ITGA2B* and *ITGB3*, are located on the long arm of chromosome 17 at q21 → 23 (Sosnoski et al. 1988) with *ITGA2B* at least 365 kb centromeric from *ITGB3* (Thornton et al. 1999). *ITGA2B* spans ≈18 kb and consists of 30 exons ranging in size from 46 to 220 bp (Heidenreich et al. 1990). Like other megakaryocyte-specific genes, its 5′ flanking region lacks TATA or CAAT boxes but contains a linear array of regulatory elements including two motifs recognized by the GATA-1 transcription factor and its cofactor FOG (Martin et al. 1993; Gaines et al. 2000),

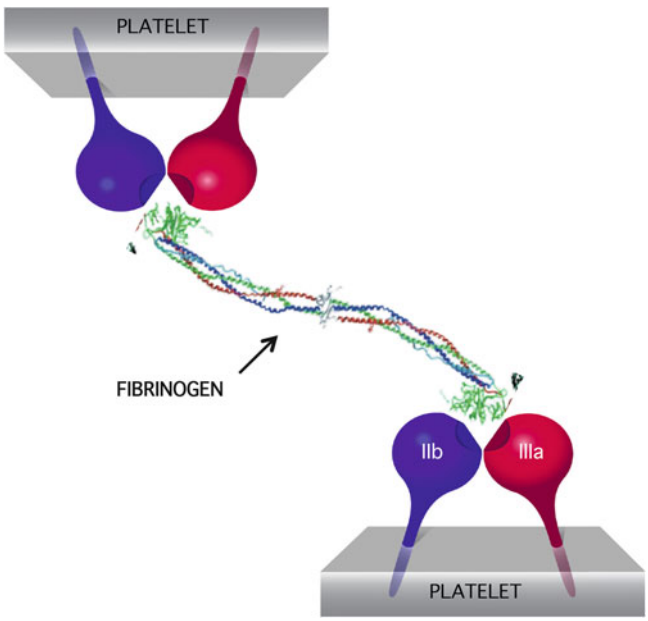

Fig. 1 Mechanism of $\alpha_{IIb}\beta_3$-mediated platelet aggregation. Platelet agonists initiate intracellular signals that convert inactive $\alpha_{IIb}\beta_3$ on resting platelets to its active conformation. This opens the binding site for macromolecular ligands such as fibrinogen in the $\alpha_{IIb}\beta_3$ headpiece. Fibrinogen then binds to the active $\alpha_{IIb}\beta_3$ on adjacent platelets, cross-linking platelets into aggregates. In the figure, α_{IIb} is designated IIb and β_3 is designated IIIa. The figure is modified from Yin et al. (2007)

sequences flanking the GATA-1 binding sites that are recognized by Fli-1 and perhaps other members of the Ets family of transcription factors (Wang et al. 2002; Jackers et al. 2004), and an Sp1-binding silencer element located between the GATA-1 sites (Shou et al. 1998). *IGTB3* spans 63 kb and contains 15 exons (Zimrin et al. 1990). Like *ITGA2B*, its 5′ flanking region lacks TATA or CAAT boxes (Villa Garcia et al. 1994). Although the transcription factors HOXA10 and Foxc2 have been reported to regulate *IGTB3* expression in myeloid (Bei et al. 2007) and endothelial (Hayashi et al. 2008) cells, respectively, there is no information about their role in megakaryocytes.

Structure of the $\alpha_{IIb}\beta_3$ Extracellular Domains

Platelets circulate in a milieu containing fibrinogen, the principle $\alpha_{IIb}\beta_3$ ligand, whose concentration in plasma is 100-fold greater than its dissociation constant for activated $\alpha_{IIb}\beta_3$ (Bennett and Vilaire 1979). Thus, the $\alpha_{IIb}\beta_3$ on circulating platelets is maintained in an inactive conformation to prevent the spontaneous formation of platelet aggregates. However, when platelets encounter a damaged blood vessel, the $\alpha_{IIb}\beta_3$ on their surfaces is almost instantaneously converted to its active conformation. This essential feature of $\alpha_{IIb}\beta_3$ function is only partially understood.

Insight into the changes in $\alpha_{IIb}\beta_3$ structure that enable it to bind soluble ligands has been provided by the solution of high-resolution crystal structures for the extracellular domains of $\alpha_{IIb}\beta_3$ and the related integrin $\alpha_v\beta_3$ (Xiao et al. 2004; Xiong et al. 2001; Zhu et al. 2008) (Fig. 2). Surprisingly, these crystal structures, thought to represent the structures of inactive $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, reveal that the amino-terminal headpieces of both extracellular domains are severely bent over two nearly parallel extracellular stalks (Xiong et al. 2001). The amino-termini of α_{IIb} and α_v consist of seven contiguous repeats that fold into β-propeller configurations (Springer 1997; Xiong et al. 2001). Each blade of the propeller is formed from four antiparallel β strands; loops connecting the strands are located on either the upper or the lower surface of the propeller. The β-propellers are then followed by a "thigh" and two "calf" domains, constituting the α subunit stalk. A knee or "genu" located between the thigh and first calf domain is the point at which α subunit bending occurs. The β_3 headpiece consists of a series of tandem nested domains: a βA domain whose fold resembles that of integrin α subunit I-domains inserted into a "hybrid" domain whose fold is similar to an I-set Ig domain inserted into a PSI (plexin, semaphorin, integrin) domain containing the β_3 amino-terminus (Xiong et al. 2004; Xiao et al. 2004). The carboxyl-terminus of the PSI domain is continuous with four tandem EGF-like repeats constituting the β_3 stalk, followed by a carboxyl-terminal βTD domain (Zhu et al. 2008). The α_{IIb} and α_v β-propellers interact with the β_3 βA domain at an interface resembling the interface between the Gα and Gβ subunits of G proteins to form the complete $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ headpieces (Xiao et al. 2004). The bent conformation of the extracellular domains is maintained by insertion of the third and fourth EGF-like β_3 domains into a crevice formed by the upper α leg on one side and the upper and lower α subunit leg on the other and with the β-propeller and βA domains forming the back of the crevice (Zhu et al. 2008).

Subsequent crystal structures of putative active $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ headpieces reveal that they are "open" because of a 62° angle of separation between the α and β subunits that results from a 10 Å downward movement of the α7 helix of the βA domain relative to the hybrid and PSI domain. This

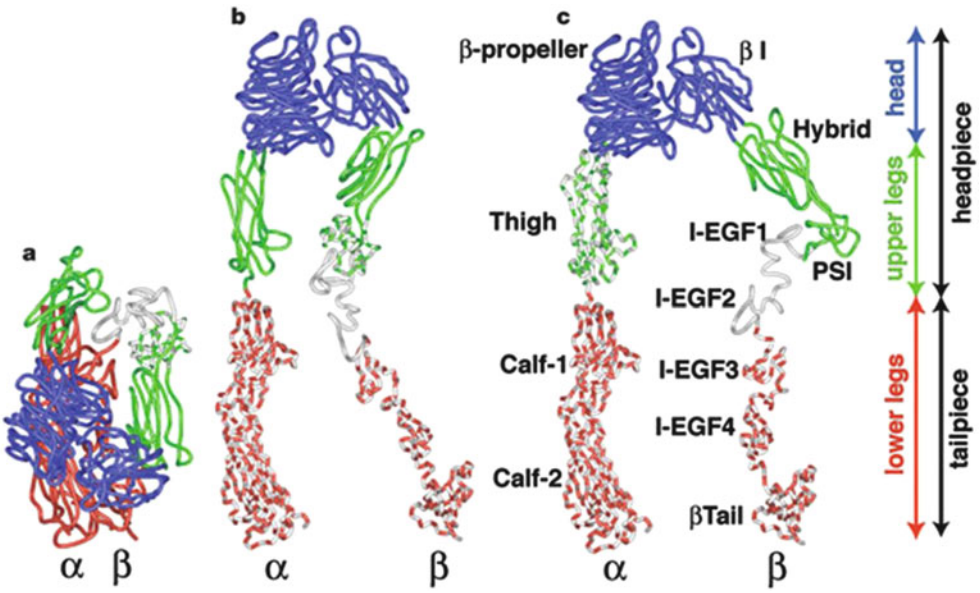

Fig. 2 The domain structure of the $\alpha_{IIb}\beta_3$ extracellular domain as derived from solved crystal structures and visualized by electron microscopy. (**a**) The bent structure of inactive $\alpha_{IIb}\beta_3$. (**b**) Extended $\alpha_{IIb}\beta_3$ with a closed headpiece. (**c**) Extended $\alpha_{IIb}\beta_3$ with an extended and open headpiece. Adapted from Xiao et al. (2004) with permission

causes reorganization of hydrogen bonds in the interface between the α7 helix and the βC strand of the hybrid domain, allowing the hybrid domain and the rigidly connected PSI domain to "swing out" (Takagi et al. 2002a; Xiao et al. 2004). In turn, this causes a 70 Å separation of the α_{IIb} and β_3 stalks at their "knees," a feature of active $\alpha_{IIb}\beta_3$ observed in rotary-shadowed EM images (Litvinov et al. 2004).

Despite the concordance of the various $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ extracellular domain crystal structures, their relevance to the structures of full-length $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ has been controversial. Negatively stained electron microscope (EM) images of active and inactive full-length $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, either free in solution or embedded in phospholipid nanodiscs, suggest that the bent conformation present in the crystals corresponds to low-affinity $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ and the shift to an open high-affinity conformation results from a "switchblade"-like movement to an extended (unbent) structure accompanied by a "scissor"-like separation of the α- and β-subunit stalks (Takagi et al. 2002b; Ye et al. 2010; Eng et al. 2011; Dai et al. 2015). Small angle X-ray scattering and size exclusion chromatography of Mn^{2+}-activated $\alpha_{IIb}\beta_3$ (Eng et al. 2011) support this suggestion, as does the ability to engineer an inactivating disulfide bond between a cysteine introduced into the β-propeller domains of α_v and α_{IIb} and a cysteine introduced into the first EGF-like domain of β_3, indicating that these regions are in proximity in inactive integrin heterodimers (Takagi et al. 2002b). On the other hand, it has been suggested that the bent conformation present in the crystals resulted from flexibility at the α_v and β_3 genus and/or from crystal contacts not likely to occur in nature (Xiong et al. 2001). This was supported by cryo-EM reconstructions of full-length presumably inactive $\alpha_{IIb}\beta_3$ that revealed an unbent structure consisting of a large globular head and an L-shaped stalk whose axis was rotated ≈60° with respect to the head and connected at an angle of ≈90° to a rod containing the α_{IIb} and β_3 TM domains (Adair and Yeager 2002). It has also been suggested that extension is a post-ligand binding event and the transition from an inactive to an active conformation results from the removal of "deadbolt" represented by the CD loop of the β_3 βTD domain locked into the βA domain (Xiong et al. 2003). However, more recent cryo-EM reconstructions of $\alpha_{IIb}\beta_3$ inserted in nanodiscs suggest that inactive $\alpha_{IIb}\beta_3$ is indeed bent, although the reconstructions differ with regard to the orientation of the $\alpha_{IIb}\beta_3$ headpiece away (Ye et al. 2010) or toward (Choi et al. 2013) the membrane surface.

$\alpha_{IIb}\beta_3$ Activation

The conversion of $\alpha_{IIb}\beta_3$ to its extended conformation is an essential event for $\alpha_{IIb}\beta_3$ activation. However, extended $\alpha_{IIb}\beta_3$ is not necessarily capable of binding ligands (Takagi et al. 2002b) since "opening" of its ligand binding site is also required. $\alpha_{IIb}\beta_3$ activation results from signals originating in the platelet cytosol that are transmitted across the plasma membrane. Moreover, truncated integrins lacking TM and cytoplasmic domains are constitutively active (Dana et al. 1991; Peterson et al. 1998; Mehta et al. 1998). Thus, specific intramolecular interactions involving their cytoplasmic (Hughes et al. 1996; Vinogradova et al. 2002) and/or TM domains constrain basal integrin activity.

Intramolecular Interactions Involving the α_{IIb} and β_3 Cytoplasmic Domains

$\alpha_{IIb}\beta_3$ can be converted from its inactive to its active form by truncating the α_{IIb} or β_3 cytoplasmic domains immediately distal to the conserved membrane-proximal α_{IIb} sequence GFFKR or the conserved membrane-proximal β_3 sequence LLITIHD (O'Toole et al. 1994) (Table 1). Substituting Ala for Arg in GFFKR or for Asp in LLITIHD has the same effect, whereas interchanging these residues does not (Hughes et al. 1996). This suggests that the membrane-proximal cytoplasmic domain sequences interact to form an activation-constraining "clasp," a feature of which is an electrostatic interaction (Metcalf et al. 2010) or salt bridge (Hughes et al. 1996) between α_{IIb} R995 and β_3 D723. Surprisingly, biophysical experiments designed to detect the clasp have generated substantially different structures, and none have directly detected an α_{IIb} R995-β_3 D723 salt bridge (Haas and Plow 1996; Vallar et al. 1999; Weljie et al. 2002; Vinogradova et al. 2004). Further, neither Li et al. (2001b) nor Ulmer et al. (2001) detected the spontaneous

Table 1 Amino acid sequences of the cytoplasmic and transmembrane domains of α_{IIb} and β_3[a]

Cytoplasmic domains	
α_{IIb}	K_{989}VGFFKRNRPPLEEDDEEGE$_{1008}$
β_3	K_{716}LLITIHDRKEFAKFEEERARAKWDTANNPLYKEATSTFTNITYRGT$_{762}$
Transmembrane domains	
α_{IIb}	W_{968}VLVGVLGGLLLLTILVLAMW$_{988}$
β_3	I_{693}LVVLLSVMGAILLIGLAALLIW$_{715}$

[a]The amino acids in the α_{IIb} and β_3 cytoplasmic and transmembrane domains are designated in the single letter code; the *subscripted numbers* correspond to the position of the preceding amino acid in the sequence for mature α_{IIb} and β_3 (Poncz et al. 1987; Zimrin et al. 1988). The αIIb GxxxG motif, β_3 G708, the membrane-proximal regions of both cytoplasmic domains, and both β_3 NxxY motifs are *underlined*

heteromeric association of peptides corresponding to the TM and cytoplasmic domains of α_{IIb} and β_3. On the other hand, the NMR structure for a disulfide-linked $\alpha_{IIb}\beta_3$ cytoplasmic domain construct (Metcalf et al. 2010) indicated that β_3 residues Lys716 and Ile719 interact directly with α_{IIb}, but a salt bridge between R995 and D723 was not identified (Fig. 3). Nonetheless, this result, coupled with the consequences of mutating the membrane-proximal α_{IIb} or β_3 cytoplasmic domains, is strong evidence that heteromeric cytoplasmic domain interactions play a role in regulating $\alpha_{IIb}\beta_3$ activity.

Ensembles of NMR structures for the β_3 cytoplasmic domain either alone or complexed with the α_{IIb} cytoplasmic domain revealed that the β_3 cytoplasmic domain is structured and composed by three helices, a membrane-proximal helix contiguous with the β_3 TM domain and two distal helices that pack together in a helix-turn-helix motif (Li et al. 2002; Metcalf et al. 2010). By contrast, while the membrane-proximal α_{IIb} cytoplasmic domain is helical, the portion distal to Asn996 is intrinsically unstructured (Metcalf et al. 2010). The two distal β_3 helices are both amphiphilic and dynamic and are stabilized by interacting with membrane bilayers. Thus, in resting platelets, hydrophobic interactions likely drive the association of the helices with the inner leaflet of the platelet plasma membrane. However, because of their dynamic nature, they likely make excursions into non-helical conformations in the aqueous cytoplasmic environment where they can interact with cytoplasmic proteins.

Heteromeric Interaction of the α_{IIb} and β_3 TM Domains

Although TM helices function as hydrophobic anchors for membrane proteins, they also commonly mediate protein oligomerization via specific sequence motifs (Moore et al. 2008). A sequence motif consisting of two glycine residues separated by one helical turn, designated GxxxG where x is any amino acid, for example, is the most overrepresented sequence motif in TM domain databases (Senes et al. 2000). GxxxG mediates the stable association of glycophorin A homodimers in red cell membranes by forming a tightly packed surface of complementary ridges and grooves (Lemmon et al. 1992; MacKenzie et al. 1997; Russ and Engelman 2000). It is noteworthy that the α_{IIb} TM helix contains the canonical GxxxG motif and the β_3 helix contains a similar motif consisting of two amino acids with small side chains separated by one helical turn (SxxxA) (Table 1). Thus, the presence of two well-characterized oligomerization motifs in the α_{IIb} and β_3 TM helices has suggested that the helices function to constrain basal $\alpha_{IIb}\beta_3$ activity.

$\alpha_{IIb}\beta_3$ becomes constitutively active when G708 in the β_3 TM domain (Li et al. 2003) or G972 or G976 in the α_{IIb} TM domain (Li et al. 2004, 2005) are replaced with polar Asn residues, consistent with the presence of an $\alpha_{IIb}\beta_3$ TM domain heterodimer when $\alpha_{IIb}\beta_3$ is inactive and its absence when $\alpha_{IIb}\beta_3$ is active (Li et al. 2003). Of two plausible explanations for the effect of the Asn replacements, it is more likely that introducing a bulky Asn residue into a closely packed $\alpha_{IIb}\beta_3$ TM domain heterodimer physically "pushes" apart the heterodimer, while on the other hand the TM domains are "pulled" into homo-oligomers when the Asn replacements form a hydrogen bonding network that neutralizes their polarity in the hydrophobic membrane core (Li et al. 2005). Regardless, these experiments provide compelling evidence that a heterodimeric TM domain interaction constrains $\alpha_{IIb}\beta_3$ in a low-affinity state and that disrupting the association of the TM domains is sufficient to cause $\alpha_{IIb}\beta_3$ activation. A number of functional and structural observations support this conclusion. First, although

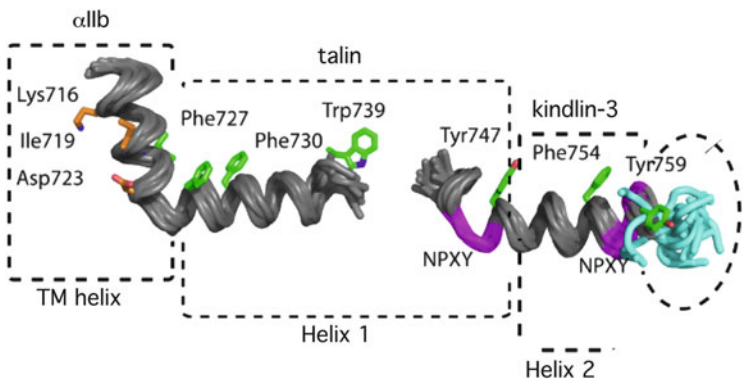

Fig. 3 Structure of the β_3 cytoplasmic domain as determined by NMR. Twenty structures in the NMR ensemble, consisting of two regions encompassing residues Leu713-Trp739 and Asp740-Thr762, were aligned separately over well-ordered regions. Domains interacting with α_{IIb}, talin, kindlin-3, and Src kinase are enclosed by *dashed lines* and *side chains* that make up interfaces are depicted as sticks. The two β_3 NPXY motifs are colored *magenta* and the Src-binding RGT motif is colored *cyan*. Adapted from Metcalf et al. (2010)

α_{IIb} or β_3 TM domain peptides with or without contiguous cytoplasmic domains readily associate homomerically (Li et al. 2001a, 2004), their homomeric association is disrupted when the complementary α_{IIb} or β_3 TM domain peptide is co-expressed (Berger et al. 2010). Second, scanning the TM helices of full-length recombinant $\alpha_{IIb}\beta_3$ with cysteine residues resulted in the formation of disulfide bonds with a helical periodicity, consistent with the presence of a unique α_{IIb}/β_3 TM heterodimer (Luo et al. 2004). Third, an ensemble of NMR structures for α_{IIb} and β_3 TM domain peptides reconstituted in phospholipid bicelles indicated that the peptides can associate to form heterodimers (Lau et al. 2009). A feature of the heterodimer is an "outer membrane clasp," a region of helix-helix interaction containing the α_{IIb} GxxxG motif and β_3 residue G708, which enforces a right-handed 25° crossing angle of the α_{IIb} and β_3 helices. The structures also identify an "inner membrane clasp" featuring hydrophobic interactions involving α_{IIb} residues F992 and F993 and an electrostatic interaction between α_{IIb} R995 and β_3 D723. However, it is possible that the inner membrane clasp is located in the cytoplasm because it is distal to the WK sequences that signify the C-terminal extent of the α_{IIb} and β_3 TM helices (Li et al. 2001a). Because mutations designed to alter the 25° crossing angle between the α_{IIb} and β_3 TM domains cause $\alpha_{IIb}\beta_3$ activation, maintaining this crossing angle is important to preserve the integrity of the outer and inner membrane clasps (Kim et al. 2012a, b).

The NMR structure of the $\alpha_{IIb}\beta_3$ TM heterodimer indicates that the GxxxG motif in α_{IIb} is located in the interface of the heterodimer. Indeed, scanning mutagenesis of the α_{IIb} and other integrin α subunit TM domains with Leu and Ala residues has confirmed that a small residue-XXX-small residue-XXX-large residue motif (Gly_{972}-xxx-Gly_{976}-xxx-Leu_{980} in α_{IIb}) represents the α subunit component of the helical interface (Berger et al. 2010). Unexpectedly, the β subunit component consisted of the converse of the α motif: a large residue-xxx-large residue-xxx-small residue motif (Val_{700}-XXX-Ile_{704}-XXX-Gly_{708} in β_3) (Berger et al. 2010). This TM helix packing arrangement juxtaposes the β_3 VxxxIxxxG motif and the α_{IIb} GxxxGxxxL motif, resulting in a closely packed and energetically favorable zipper-like interface that is conserved across the entire integrin family (Fig. 4). The interface is demonstrably weaker than the canonical glycophorin A GxxxG homodimerization motif (MacKenzie et al. 1997) and likely has evolved to support helix-helix interactions whose strength is appropriate for a system like $\alpha_{IIb}\beta_3$ that must undergo rapid conformational switching.

Protein Binding to the β_3 Cytoplasmic Domain Causes $\alpha_{IIb}\beta_3$ Activation

Remarkably, considering their short lengths, the α_{IIb} and β_3 cytoplasmic domains have been reported to interact with a large number of cytosolic proteins (Buensuceso et al. 2004). Although the significance of most of these interactions is uncertain, binding of the FERM (four-point-one, ezrin, radixin, moesin) domains of the cytoskeletal protein talin-1 and the focal adhesion protein kindlin-3 to the β_3 cytoplasmic domain is necessary for physiologic $\alpha_{IIb}\beta_3$ activation.

Talin, an abundant 250-kDa cytoskeletal protein, binds to the cytoplasmic domain of integrin β subunits as well as to cytoskeletal proteins such as actin and vinculin (Calderwood 2004). Of the two talin isoforms, talin-1 is the predominant platelet isoform and is present in the platelet cytoplasm as an antiparallel homodimer (Critchley 2009; Goult et al. 2013). The talin-1 monomer is composed of a 50-kDa head domain (THD) containing its principal integrin binding site and a 220-kDa rod domain that binds to integrins with lower affinity (Kloeker et al. 2004). The THD is an ≈300-residue

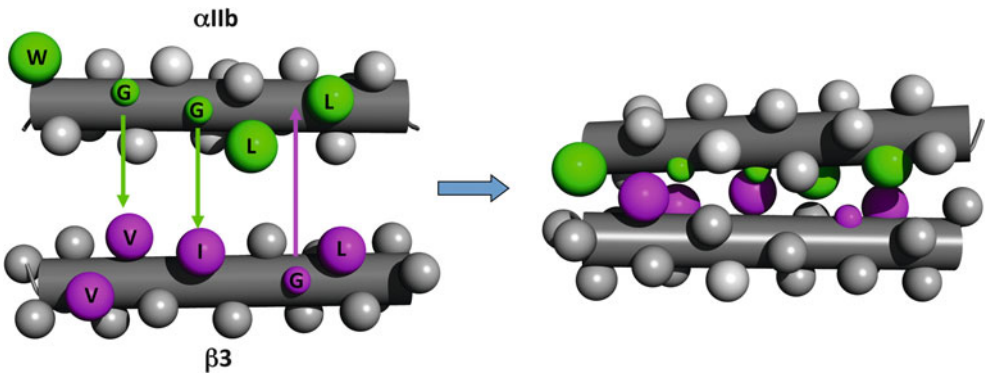

Fig. 4 Model of the reciprocal "large-small" integrin TM heterodimer interface. The integrin heterodimer is represented as an idealized pair of helices with *large spheres* denoting large hydrophobic residues and *small spheres* representing small polar residues. *G* glycine, *L* leucine, *V* valine, *I* isoleucine, and *W* tryptophan. Adapted from Bennett, J.S., and Moore, D.T. *Haematologica*. 2010; 95:1049–1051. Regulation of platelet β_3 integrins. Obtained from the Haematologica Journal website http://www.haematologica.org

FERM domain that folds into F0, F1, F2, and F3 subdomains (Kloeker et al. 2004); both F2 and F3 bind to the β_3 cytoplasmic domain (Moore et al. 2012), although the affinity of F3 binding is substantially greater (Calderwood et al. 2002). In resting platelets, the β_3 binding site in the F3 subdomain is masked by sequences located in the rod domain (Goult et al. 2013). A structural model of the talin-1 dimer that accounts for this auto-inhibition revealed that the dimer is folded into a donut shape with the two rods forming the donut and the two heads packed into the central donut hole (Goult et al. 2013). How talin auto-inhibition is relieved after platelet stimulation remains a fundamental problem in understanding physiologic $\alpha_{IIb}\beta_3$ activation. The enzyme calpain cleaves talin, releasing its head domain (Yan et al. 2001), but calpain activation in platelets occurs relatively late after platelet stimulation (Fox et al. 1993) and would be unlikely to contribute to initial $\alpha_{IIb}\beta_3$ activation. More likely possibilities include talin activation after binding to membrane-associated phosphoinositol 4,5-bisphosphate, an interaction previously found to enable talin binding to the β_1 cytoplasmic domain (Martel et al. 2001); the formation of a complex containing talin, the low molecular weight GTP binding protein Rap-1, and RIAM (Rap1-GTP interacting adapter molecule) that can deliver talin to the platelet plasma membrane to bind to β_3 (Lee et al. 2009) [surprisingly, $\alpha_{IIb}\beta_3$ function was found to be normal in a RIAM knockout mouse (Stritt et al. 2015)]; and the agonist-stimulated transfer of talin $\pm$ kindlin to β_3 via the scaffolding protein ADAP (adhesion and degranulation promoter protein) (Kasirer-Friede et al. 2014).

In vitro, THD binding to the proximal β_3 cytoplasmic domain helix is sufficient to cause $\alpha_{IIb}\beta_3$ extension and activation (Ye et al. 2010). Importantly, in vivo, the platelet-specific deletion of talin-1 in mice also either profoundly impairs (Petrich et al. 2007) or abrogates (Nieswandt et al. 2007) ligand binding to $\alpha_{IIb}\beta_3$ and platelet aggregation. THD binding to the β_3 cytoplasmic domain helix occurs in two steps (Calderwood et al. 2002; Wegener et al. 2007; Anthis et al. 2009) (Fig. 5). In the first step, the F3 subdomain binds to the β_3 NPLY motif and to β_3 W739 in a hydrophobic interaction that resembles canonical PTB domain binding to ligands (Garcia-Alvarez et al. 2003). This interaction is necessary but not sufficient to cause $\alpha_{IIb}\beta_3$ activation (Wegener et al. 2007). A second step is required in which β_3 residues F727 and F730 are buried in the F3 subdomain, thereby enabling $\alpha_{IIb}\beta_3$ activation when a salt bridge between talin-1 residue K324 and β_3 D723 competes with the electrostatic interaction between β_3 D723 and α_{IIb} R995 (Anthis et al. 2009) to initiate the separation of the β_3 and α_{IIb} cytoplasmic and TM domains.

Given the evidence that talin alone is sufficient to activate $\alpha_{IIb}\beta_3$ in vitro, it was perplexing that patients with leukocyte adhesion deficiency (LAD) type III—whose blood cells specifically lack the focal adhesion protein kindlin-3 but contain normal amounts of talin—manifest a bleeding disorder resembling Glanzmann thrombasthenia, as well as perturbed β_2 integrin-mediated leukocyte function (Moser et al. 2008, 2009a, b). Kindlin-3 is the third member of a three-member family identified initially because mutations in the first family member, kindlin-1, cause the Kindler syndrome, an

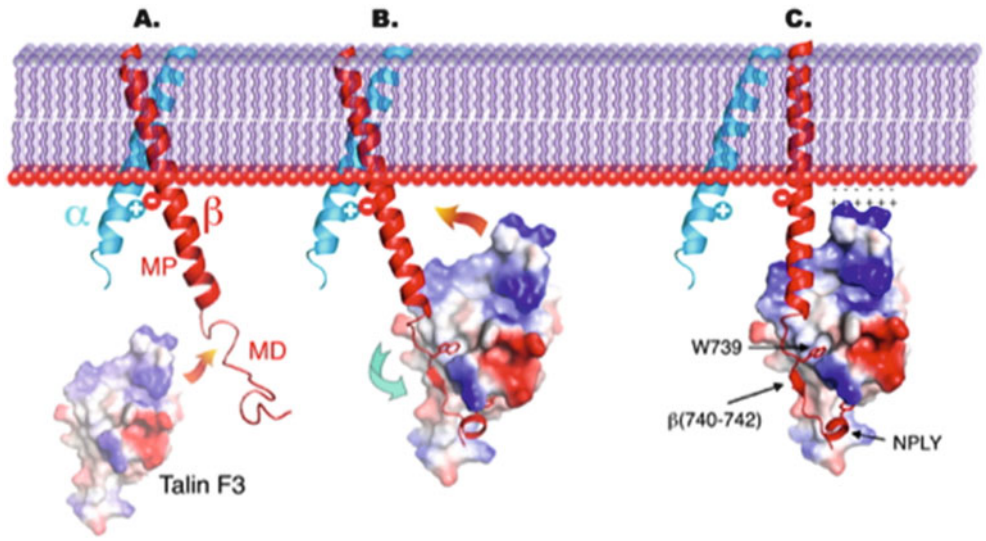

Fig. 5 Model of talin-induced integrin activation. (**a**) The talin F3 subdomain, no longer auto-inhibited, is available to bind to the β_3 cytoplasmic domain. (**b**) F3 engages its membrane-distal β_3 cytoplasmic domain binding site that includes an NPLY motif as well as W739, but does not cause $\alpha_{IIb}\beta_3$ activation. (**c**) F3 now engages the membrane-proximal of the β_3 cytoplasmic domain, disrupting the electrostatic interactions between the membrane-proximal portions of the α_{IIb} and β_3 cytoplasmic domains and causing separation of the α_{IIb} and β_3 transmembrane domains. In turn, this results in $\alpha_{IIb}\beta_3$ extension and activation (not shown). Adapted from Wegener et al. (2007) with permission from Elsevier Inc.

autosomal recessive blistering skin disease affecting actin attachment in basal keratinocytes (Karakose et al. 2010). Kindlin-3 is a 76-kDa protein whose FERM domain, like the talin FERM domain, contains F0, F1, F2, and F3 subdomains. But unlike talin, its F2 domain contains an inserted PH domain that facilitates the interaction of kindlin-3 with membranes (Karakose et al. 2010; Liu et al. 2011). Kindlin-3 binds to the β_3 cytoplasmic domain via its FERM domain, but at a site distinct from the site bound by talin (Bledzka et al. 2012; Yates et al. 2012). Although kindlin-3 binding to β_3 alone is not sufficient to cause $\alpha_{IIb}\beta_3$ activation, agonist-stimulated $\alpha_{IIb}\beta_3$ activation in platelets does not occur when kindlin-3 is absent (Moser et al. 2008). Kindlin-3 and talin-1 are present in essentially stoichiometric amounts in mouse platelets (Klapproth et al. 2015). However, in mice hypomorphic for kindlin-3 gene expression, $\alpha_{IIb}\beta_3$ activity can be titrated by varying the amount of kindlin-3, with 5–10 % of the wild-type amount sufficient to support basal platelet function (Klapproth et al. 2015). These observations are consistent with the idea that kindlin-3 plays a permissive role in enabling talin to activate $\alpha_{IIb}\beta_3$ (Fig. 6). How this occurs is not known. It has been suggested that kindlin-3 does not enhance $\alpha_{IIb}\beta_3$ activation but rather increases the avidity of active $\alpha_{IIb}\beta_3$ for multivalent, but not univalent ligands, by promoting $\alpha_{IIb}\beta_3$ clustering (Ye et al. 2013). However, kindlins are monomeric (Calderwood et al. 2013) so their ability to promote $\alpha_{IIb}\beta_3$ clustering would have to be indirect and additional kindlin binding partners such as migfilin (Brahme et al. 2013) and ILK (Bottcher et al. 2009; Jones et al. 2014) would be required. A second possibility is that kindlin-3 binds transiently to the distal β_3 cytoplasmic domain to expose and stabilize the more proximal talin binding site (Metcalf et al. 2010). Thus, kindlin might bind simultaneously with talin in a positively cooperative manner, although it has not yet been possible to trap a putative ternary complex.

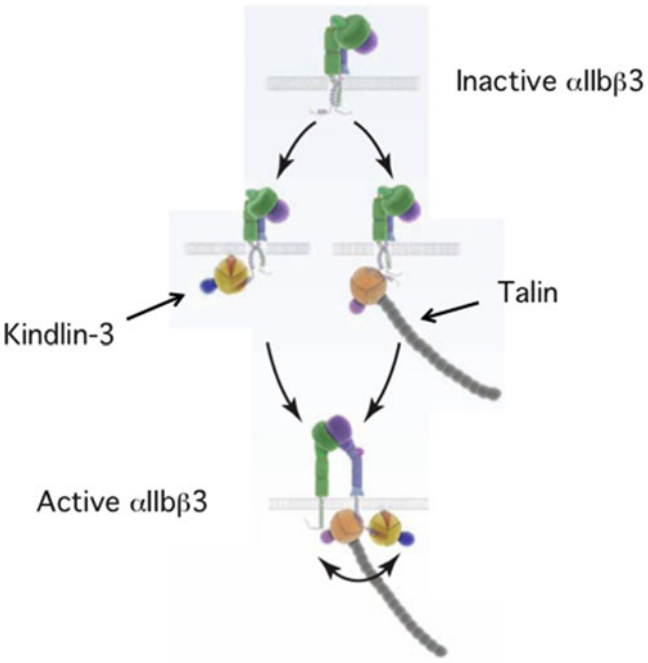

Fig. 6 Binding of both talin-1 and kindlin-3 are required to cause physiologic $\alpha_{IIb}\beta_3$ activation. Talin-1 and kindlin-3 bind to separate sites on the β_3 cytoplasmic tail. It is not known whether binding is simultaneous or sequential, and if it is the latter, what is the order of binding. Adapted from Moser et al. (2009b). Reprinted with permission from AAAS

$\alpha_{IIb}\beta_3$-Mediated Outside-In Signaling in Platelets

$\alpha_{IIb}\beta_3$-mediated "outside-in" signaling is initiated by the trans-autophosphorylation of the tyrosine kinase c-Src bound via its SH3 domain to the β_3 cytoplasmic domain of active $\alpha_{IIb}\beta_3$ (Arias-Salgado et al. 2003; Senis et al. 2014). In turn, activated c-Src initiates a signaling cascade resulting in platelet spreading on fibrinogen-coated surfaces and the retraction of fibrin clots (Shattil et al. 1998; Haling et al. 2011). The c-Src SH3 domain binds to the carboxyl-terminal β_3 sequence Arg-Gly-Thr (RGT), an interaction inhibited by proline-rich peptides selective for SH3 domains (Arias-Salgado et al. 2003). However, the carboxyl-terminus of β_3 is quite different from the polyproline sequences recognized by SH3 domains (Metcalf et al. 2010; Li 2005). Moreover, inactive c-Src adopts a compact structure that is stabilized by intramolecular interactions including binding of the linker connecting its SH2 and kinase domains to the polyproline binding site in the SH3 domain. Thus, in resting platelets, there is little or no specific interaction between β_3 and c-Src. However, following agonist-stimulated platelet activation, c-Src is "unlatched" by dephosphorylation of pTyr530 (Arias-Salgado et al. 2005), and the β_3 cytoplasmic domain is now able to bind to SH3 domain residues 98–103 vacated by the linker between the c-Src SH2 and kinase domains. Subsequent $\alpha_{IIb}\beta_3$ clustering increases the concentration of β_3-bond c-Src, enabling the completion of c-Src autotransphosphorylation and the initiation of outside-in platelet signaling.

The G-protein subunit $G\alpha_{13}$ has also been reported to bind to an ExE motif ($E_{731}E_{732}E_{733}$ in β_3) in the proximal β_3 cytoplasmic domain and to facilitate in some way the phosphorylation of β_3-bound c-Src (Gong et al. 2010; Shen et al. 2013). $G\alpha_{13}$ and the THD appeared to compete for binding to β_3 such that there were reciprocal waves of talin and $G\alpha_{13}$ binding with talin bound during inside-out signaling and $G\alpha_{13}$ bound during outside-in signaling. This suggested that talin and $G\alpha_{13}$ binding might act as a molecular switch to modulate the direction of $\alpha_{IIb}\beta_3$-mediated signaling.

Ligand Binding to $\alpha_{IIb}\beta_3$

Fibrinogen, the major $\alpha_{IIb}\beta_3$ ligand, is composed of pairs of Aα, Bβ, and γ chains folded into three nodular domains. Although fibrinogen, like many other integrin ligands, contains canonical RGD motifs, binding of soluble fibrinogen to active $\alpha_{IIb}\beta_3$ is mediated by the sequence located at the carboxyl-terminus of the fibrinogen γ chain (Farrell et al. 1992). Nevertheless, both carboxyl-terminal γ chain peptides (Kloczewiak et al. 1983) and peptides containing an RGD motif inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ (Gartner and Bennett 1985). However, competitive binding measurements have revealed that γ chain and RGD peptides cannot bind simultaneously to $\alpha_{IIb}\beta_3$, suggesting that they either bind to the same or overlapping sites or that binding of one peptide induces a conformational change in the $\alpha_{IIb}\beta_3$ headpiece that prevents binding of the other (Bennett et al. 1988).

Crystal structures of the $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ headpieces containing γ chain and RGD peptides have provided a structural explanation for these puzzling observations. The binding site for fibrinogen on the $\alpha_{IIb}\beta_3$ headpiece is located at the interface of the β_3 βA and α_{IIb} β-propeller domains and consists of a "specificity-determining" loop in the β_3 βA domain and a "cap" composed of four loops linking blades 2 and 3 and blades 3 and 4 on the upper surface of the β-propeller domain (Xiao et al. 2004). The structures confirm suggestions based on the consequences of β-propeller mutations that fibrinogen binds to the upper surface of the β-propeller in a region centered around the third amino-terminal repeat (Tozer et al. 1999; Basani et al. 2000; Kamata et al. 2001). It is noteworthy, however, that the intact fibrinogen molecule binds to $\alpha_{IIb}\beta_3$ with an affinity several orders of magnitude greater than the affinity of peptides. Thus, crystal structures solved in the presence of peptides may not completely account for fibrinogen binding.

Fibrinogen binding to $\alpha_{IIb}\beta_3$ requires divalent cations (Bennett and Vilaire 1979). Although there are eight divalent cation-binding sites in the $\alpha_{IIb}\beta_3$ crystal structure (Xiong et al. 2001), only the three located in a linear array in the βA domain participate in ligand binding (Chen et al. 2003). The middle site, the βA MIDAS (metal ion-dependent adhesion site), coordinates Mg^{2+}, whereas the flanking sites, ADMIDAS (adjacent to MIDAS) and SyMBS (synergistic metal binding site), normally contain Ca^{2+}. When fibrinogen γ chain peptides were soaked into $\alpha_{IIb}\beta_3$ headpiece crystals, they bound in a groove in the interface between the β3 βA and α_{IIb} β-propeller domains (Springer et al. 2008). Like the Asp of RGD, the side chain of the penultimate γ chain peptide residue, Asp_{410}, coordinated with the MIDAS Mg^{2+} and the free carboxyl of carboxyl-terminal Val_{411} coordinated with the ADMIDAS Ca^{2+} through a water molecule as well. The γ chain peptide also made hydrophobic contacts with α_{IIb} residues located in the loops connecting β-propeller blades 2 and 3 and blades 3 and 4. Lastly, like the Arg of RGD, γ chain residue Lys_{406} made a charged hydrogen bond with α_{IIb} residue Asp_{224}. Thus, the binding sites for γ and RGD peptides overlap, but the γ chain binding site is substantially larger.

Although RGD-containing peptides are $\alpha_{IIb}\beta_3$ antagonists, preincubating $\alpha_{IIb}\beta_3$ with these peptides can surprisingly cause $\alpha_{IIb}\beta_3$ activation (Du et al. 1991). Soaking increasing concentrations of the peptide GRGDSP into $\alpha_{IIb}\beta_3$ headpiece crystals in the presence of various combinations of Mg^{2+}/Ca^{2+} or Mn^{2+}/Ca^{2+} has made it possible to characterize sequential changes in the headpiece that convert it from a closed low-affinity to an open high-affinity conformation (Zhu et al. 2013). The changes occur exclusively in the βA domain and eventually result in a piston-like movement of its α7 helix toward the hybrid domain, accompanied by "swing out" of the hybrid domain and headpiece opening. Repeating these experiments in the presence of the γ chain sequence AGDV rather than GRGDSP produced the same sequence of headpiece changes (Lin et al. 2016). Unlike GRGDSP, however, AGDV has little contact with αIIb, confirming the predominant role of the βA domain in modulating the conformation of the $\alpha_{IIb}\beta_3$ headpiece.

Besides interacting with fibrinogen, individual activated $\alpha_{IIb}\beta_3$ molecules attach to fibrin fibers (Dai et al. 2015) such that aggregated platelets are major components of hemostatic blood clots and thrombi (Collet et al. 2002). Further, the interaction of $\alpha_{IIb}\beta_3$ with fibrin is involved in platelet-driven clot contraction (Carr 2003; Lam et al. 2011). $\alpha_{IIb}\beta_3$ activation and fibrinogen conversion to fibrin in vivo occur almost simultaneously. Thus, while the formation of hemostatic platelet plugs and platelet thrombi is initially due to fibrinogen binding to $\alpha_{IIb}\beta_3$, it evolves into an interaction between $\alpha_{IIb}\beta_3$ and monomeric and oligomeric fibrin as fibrinogen is converted to fibrin by thrombin generated on or near the surface of activated platelets (Fig. 7). Measurements of $\alpha_{IIb}\beta_3$ binding to fibrinogen and fibrin monomer using optical trap-based force spectroscopy revealed that the binding of surface-attached fibrin and fibrinogen to $\alpha_{IIb}\beta_3$ are both quantitatively and qualitatively different (Litvinov et al. 2016). First, $\alpha_{IIb}\beta_3$ has a greater propensity to interact with monomeric fibrin than fibrinogen at the same surface density. Second, the strength of $\alpha_{IIb}\beta_3$ binding to monomeric fibrin is greater. Taken together, these observations imply that the affinity of $\alpha_{IIb}\beta_3$ for monomeric fibrin is greater than its affinity for fibrinogen. Further, fibrin lacking wild-type γ/γ chains retains $\alpha_{IIb}\beta_3$-binding activity, suggesting that the γC-terminal AGDV motif may not be the major $\alpha_{IIb}\beta_3$ binding site in fibrin, whereas it is indispensable for soluble fibrinogen binding to $\alpha_{IIb}\beta_3$ (Farrell et al. 1992). This suggestion is supported by the reduced $\alpha_{IIb}\beta_3$ binding activity of fibrin mutants lacking α chain RGD motifs. In fact, binding to these motifs in fibrin may even be preferred since the affinity of RGD for $\alpha_{IIb}\beta_3$ is greater than of AGDV

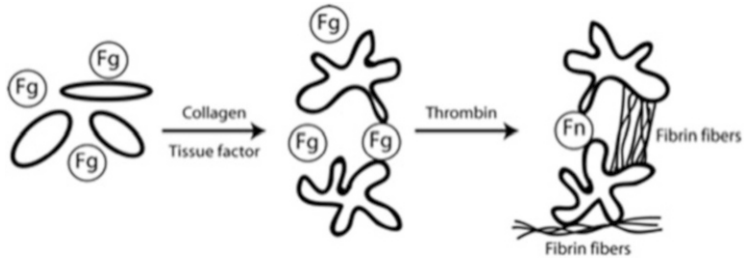

Fig. 7 Platelet aggregation initially begins with fibrinogen binding to $\alpha_{IIb}\beta_3$, but quickly evolves into $\alpha_{IIb}\beta_3$ interaction with monomeric, oligomeric, or polymeric fibrin as fibrinogen is converted to fibrin by thrombin generated at or near the surface of the activated platelets. This figure was originally published in *The Journal of Biological Chemistry*: Litvinov, R.I., Farrell, D.H., Weisel, J.W., and Bennett, J.S. The platelet integrin $\alpha_{IIb}\beta_3$ differentially interacts with fibrin versus fibrinogen. 2106; 291:7858–7867. ©The American Society for Biochemistry and Molecular Biology

(Sun et al. 2012; Kloczewiak et al. 1983). Nonetheless, the absence of RGD in the presence of free γC-dodecapeptide does not completely abrogate the interactions of fibrin with $\alpha_{IIb}\beta_3$, suggesting that additional sites in fibrin other than γAGDV, αRGDF, and αRGDS can interact with $\alpha_{IIb}\beta_3$.

Take-Home Messages

- Binding of macromolecular ligands such as fibrinogen or von Willebrand factor to the major platelet integrin $\alpha_{IIb}\beta_3$ is required for platelet aggregation.
- $\alpha_{IIb}\beta_3$ on circulating platelets is inactive, unable to bind macromolecular ligands. Platelet stimulation by agonists such as ADP and thrombin causes $\alpha_{IIb}\beta_3$ to assume its active ligand binding conformation.
- $\alpha_{IIb}\beta_3$ is a heterodimer, assembled from independently synthesized α_{IIb} and β_3 monomers in the endoplasmic reticulum. Failure to synthesize sufficient numbers of correctly folded heterodimers prevents $\alpha_{IIb}\beta_3$ expression and produces the bleeding disorder Glanzmann thrombasthenia.
- $\alpha_{IIb}\beta_3$ on circulating platelets is constrained in its inactive conformation by a clasp composed by specific intramolecular interactions involving the α_{IIb} and β_3 transmembrane domains and membrane-proximal portions of the α_{IIb} and β_3 cytoplasmic domains.
- Crystal structures of inactive $\alpha_{IIb}\beta_3$ reveal that the amino-terminal headpiece of its extracellular domain is severely bent over two nearly parallel extracellular stalks. Conversion of $\alpha_{IIb}\beta_3$ to an extended conformation is an essential event for $\alpha_{IIb}\beta_3$ activation.
- Binding of the cytoskeletal protein talin-1 and the focal adhesion protein kindlin-3 to the β_3 cytoplasmic domain causes disruption of the clasp that maintains inactive $\alpha_{IIb}\beta_3$ to initiate $\alpha_{IIb}\beta_3$ extension and activation.
- $\alpha_{IIb}\beta_3$ binds to both fibrinogen and fibrin. Fibrinogen binding to $\alpha_{IIb}\beta_3$ mediates platelet aggregation, whereas fibrin binding to $\alpha_{IIb}\beta_3$ mediates the retraction of fibrin clots.

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GPVI

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Abstract

Platelet membrane glycoprotein VI has been identified relatively recently as compared to other major platelet glycoproteins such as the GPIb-V-IX complex and integrin $\alpha_{IIb}\beta_3$. This is probably due to the poor clinical manifestations associated to a GPVI deficiency although strong evidence have been accumulated during the past 15 years that this receptor is critically involved in pathology. The role of GPVI is first to promote thrombus formation, but a rapidly increasing number of studies reveal that GPVI contributes to many processes beyond thrombosis including inflammation, host defence and cancer dissemination.

Key Discoveries

Glycoprotein VI (GPVI), initially described in 1982 as a 58–60 kDa glycosylated platelet membrane protein, was identified 5 years later as a collagen receptor on the observation that platelets from a patient with a GPVI deficiency did not respond to collagen (Clemetson et al. 1982; Sugiyama et al. 1987). Key progresses were made with the discovery that GPVI signals via the γ chain common to Fc receptors (FcRγ) and with the identification of GPVI-specific agonists, the snake venom protein convulxin and collagen-related peptides (CRP) (Tsuji et al. 1997; Ezumi et al. 1998; Jandrot-Perrus et al. 1997; Polgar et al. 1997; Morton et al. 1995; Kehrel et al. 1998). Last but not least, the cloning of GPVI at the end of the 1990s permitted to determine its structure, to produce recombinant GPVI and specific anti-GPVI antibodies, to obtain genetically modified animals and in turn to dissect the role of GPVI in physiology and pathology (Clemetson et al. 1999; Jandrot-Perrus et al. 2000). At the same time, the identification of patients with genetic or acquired GPVI deficiency showed that the absence of GPVI is not associated with severe bleeding; however results obtained in animal models implicated GPVI as having a role in arterial thrombosis. These observations led to a novel important concept that targeting GPVI could be beneficial in the prevention and treatment of thrombosis without generating a bleeding risk (Dutting et al. 2012; Zahid et al. 2012). In addition there is a growing evidence that GPVI supports platelet functions beyond haemostasis and thrombosis: this includes the contribution of platelets to inflammation (Gros et al. 2014) and to tumour metastasis (Jain et al. 2009).

GPVI Structure

GPVI is type I transmembrane protein with a 58–60 kDa molecular mass, 45 % of which is carbohydrate (Clemetson et al. 1999; Jandrot-Perrus et al. 2000). GPVI belongs to the immunoglobulin superfamily. The human gene is located on chromosome 19 within the leukocyte receptor cluster (LCR) together with leukocyte Ig-like receptors (LIRs), killer-cell Ig-like receptors (KIRs), leukocyte-associated Ig-like receptors (LAIRs) and the receptor for IgA FcαRI (Jandrot-Perrus et al. 2000). The first loop of GPVI is of the Ig constant type C2-1 type found in LILRs, while the

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second is of the C2-2 type also found in KIRs (Martin et al. 2002). LRC genes must have a common ancestor and have evolved by domain's recombination, duplication and shuffling. Although most ligands of LRC receptors are HLA class I molecules, some of them interact with non-HLA ligands and this is the case of GPVI. Interestingly, LAIR-1, which is structurally related to GPVI, also binds to collagen (Brondijk et al. 2010). However GPVI and LAIR-1 have opposite functions since GPVI is an activating receptor on platelets and LAIR-1 is an inhibitory receptor on immune cells.

The 339 amino acids (AA) sequence comprise a 21 AA signal peptide followed by the 247 AA of the extracellular portion of GPVI which harbours two immunoglobulin-like loops formed by disulphide bridges (D1 and D2) linked by an interdomain and that exposes the structural determinants for binding to collagen (Fig. 1). The crystal structure solved at 2.4 Å showed that the two loops are orientated at ~90° as described in other receptors of this family such as FcαRI (Horii et al. 2006). Combining data of crystallography, docking of collagen-related peptides, mutagenesis and epitope mapping converge to propose a model in which clusters of residues at the surface of the D1 loop and hinge region are implicated in GPVI binding to collagen: a putative collagen-binding domain (CBD) composed of a basic groove form of K41, K59, R60 and R166 is added a more distal site including hydrophobic residues L36, V34 and the N-glycan chain at N72 (Lecut et al. 2004a; Smethurst et al. 2004; Kunicki et al. 2005; Dumont et al. 2006; Horii et al. 2006; O'Connor et al. 2006). However, the recently determined crystal structure and collagen-binding surface of LAIR-1 suggest another collagen-binding mode involving GPVI residues R38, E40 and possibly W76 (Brondijk et al. 2010). In the GPVI crystal, the D2 loop is also involved in the formation of a back-to-back dimer that brings the two CBD nearly antiparallel and in a configuration allowing a simultaneous binding on two parallel collagen triple helices (Horii et al. 2006). Indeed, GPVI dimers have a high affinity for collagen while the monomer has a poor affinity (see below) (Miura et al. 2002). A highly O-glycosylated membrane proximal sequence forms a mucin-like stalk domain assumed to maintain D1–D2 at a distance from the platelet surface. Proximal to the membrane, GPVI presents a site of cleavage by metalloproteinases (Gardiner et al. 2004).

The 19 AA-transmembrane domain contains a charged residue, R252 that forms a salt bridge with D11 of the FcR-γ-chain allowing the expression of the complex at the platelet surface.

The cytoplasmic domain contains 51 AA without apparent homology with other receptor of this family and no tyrosine. The cytoplasmic tail of mouse GPVI lacks the 24 C-terminal residues of human GPVI (Jandrot-Perrus et al. 2000). Adjacent to the membrane are identified a basic sequence that stabilizes the interaction with FcRγ and a calmodulin-binding motif followed by a proline-rich domain (PRD) to which the Src family kinases, Lyn and Fyn are constitutively bound (Suzuki-Inoue et al. 2002; Bori-Sanz et al. 2003; Gardiner et al. 2004).

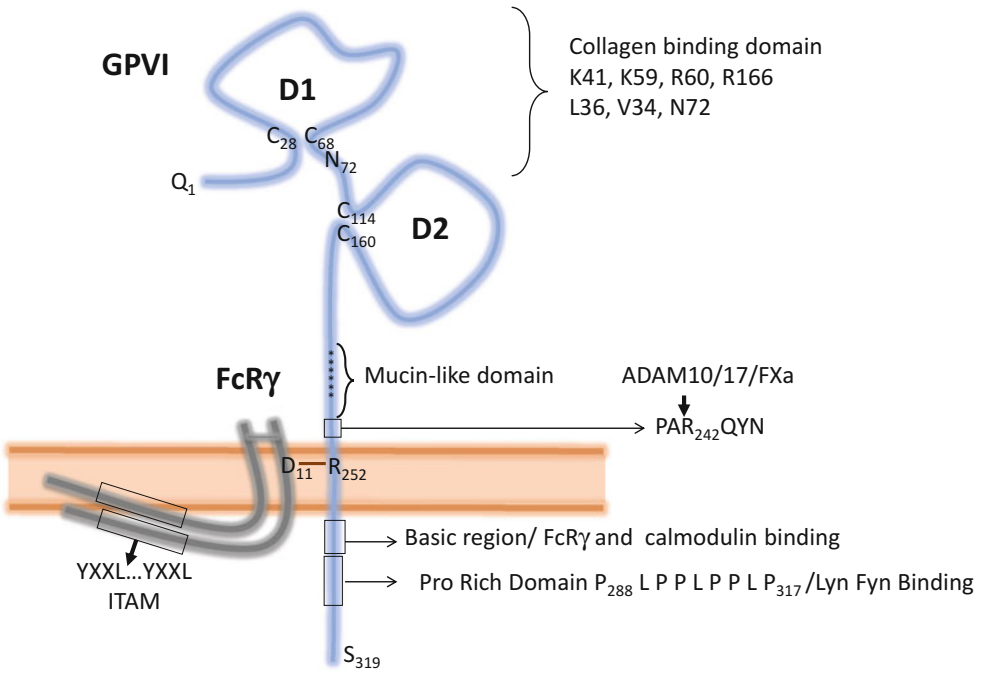

Fig. 1 Schematic representation of the GPVI-FcRγ complex. GPVI numbering starts at the first residue after the signal peptide. FcRγ chain C-terminus is represented buried in the membrane as assumed to be in resting platelets

The GPVI Receptor-Signalling Pathway

The first event in GPVI signalling is the phosphorylation of two tyrosine residues located within a conserved motif in the FcRγ chain known as immunoreceptor tyrosine-based activation motif (ITAM). Upon ligand binding, the ITAM tyrosine residues are phosphorylated by the Src family kinases (SFK). Lyn and Fyn are the major SFK mediating FcRγ phosphorylation (Ezumi et al. 1998; Quek et al. 2000; Severin et al. 2012). Binding of Lyn and Fyn via their SH3 domains to the PRD of the GPVI cytoplasmic allows them to acquire a partial activated state and bring them in contact with their substrate upon receptor ligation resulting in a fast phosphorylation (Schmaier et al. 2009). Interestingly, the protein tyrosine phosphatase CD148 has been shown to regulate Fyn and Lyn downstream of GPVI by dephosphorylating an inhibitory tyrosine residue, which holds the SFK in an inactive conformation (Senis et al. 2009) and additional yet unidentified phosphatases may contribute to regulate SFK activation. Phosphorylated ITAMs of clustered FcRγ chains provide a docking site for the tandem SH2 domains of the spleen tyrosine kinase, Syk. Binding of Syk to the phosphorylated ITAM leads to its activation through a combination of conformational change, phosphorylation by SFKs and autophosphorylation (Mocsai et al. 2010). Mouse platelets, which are deficient in Syk, are unresponsive to collagen confirming the critical role of the tyrosine kinase in platelet activation by collagen (Poole et al. 1997). Interestingly, Syk deficiency or treatments by the Syk inhibitors, fostamatinib or PRT060318, developed to treat inflammatory disease as rheumatoid arthritis or asthma as to reduce heparin-induced thrombocytopenia and immune complex-mediated thrombosis, have a minimal impact on bleeding (Law et al. 1999; Andre et al. 2011) (Table 1).

The binding and activation of Syk is a critical event in the formation of a signalosome consisting of various adapter and effector proteins. Central to the formation of the signalosome is linker for activation of T cells (LAT), a transmembrane protein localized to lipid rafts with nine conserved tyrosine residues in its cytoplasmic domain (Pasquet et al. 1999). Phosphorylated LAT recruits the adapter proteins Grb2 (growth factor receptor bound protein 2), Gads (Grb2-related adaptor protein downstream of Shc) and SLP-76 (SH2 domain containing leukocyte protein of 76 kDa) (Asazuma et al. 2000; Hughes et al. 2008). LAT also binds class I PI-3 kinases (phosphatidylinositol 3-kinase) that catalyse the formation of the phosphotinositide 3,4,5-trisphosphate (PIP3) (Gibbins et al. 1998). The PI3K regulatory subunit p85α plays a key role in GPVI signalling and the catalytic subunits p110α and β have non-redundant roles in GPVI-induced platelet activation and thrombus formation (Gilio et al. 2009). Interestingly, drugs targeting PI3Kβ do not inhibit platelet adhesion but provoke thrombus instability (Laurent et al. 2015). Altogether LAT, SLP76 and PIP3 lead to the recruitment of the phospholipase Cγ2 (PLCγ2) via its SH2 and PH domains and to its phosphorylation by kinases of the Tec family, Btk (Bruton's tyrosine kinase) and Tec, which also bind to PIP3 (Quek et al. 1998; Atkinson et al. 2003). Mice deficient in

Table 1 Effect of signalling proteins deficiency on collagen-triggered responses and haemostasis

Molecule targeted	Thrombus formation	Hemostasis	References
Fyn/lyn	Fyn−/− normal thrombus formation on collagen in vitro	Normal	Quek et al. (2000)
	Lyn−/−↓ thrombus formation on collagen in vitro		Severin et al. (2012)
	Fyn/−Lyn−/−↓ thrombus formation on collagen in vitro		
Syk	↓↓↓ Thrombus formation on collagen in vitro	Normal	Law et al. (1999)
			Andre et al. (2011)
LAT	↓ Thrombus formation on collagen in vitro	Normal	Judd et al. (2002)
			Hughes et al. (2008)
Gad	Normal thrombus formation on collagen in vitro		Hughes et al. (2008)
Vav1/2/3	Delayed occlusion time after FeCl3 carotid artery injury ↓ thrombus formation on collagen in vitro		Pearce et al. (2004)
Sip76	↓↓↓ Thrombus formation on collagen in vitro		Bezman et al. (2008)
P13K α/β	↓ Thrombus formation after FeCl3 carotid artery injury	Normal	Gilio et al. (2009)
			Laurent et al. (2015)
PLCγ2	↓↓↓ Thrombus formation on collagen in vitro	↑↑	Mangin et al. (2003)
	↓↓↓ Thrombus formation (laser injury model)		Rathore et al. (2004)
	↓↓↓ Thrombus formation on collagen in vitro		
Rac 1	↓↓↓ Thrombus formation (laser injury model)	↑	Pleines et al. (2009)
	↓↓↓ Thrombus formation (lcarotid artery ligation)		Stefanini et al. (2012)
STAT 3	↓↓ Thrombus formation on collagen in vitro		Zhou et al. (2013)

↓, mild; ↓↓, moderate; and ↓↓↓, severe defect in platelet function

PLCγ2 are protected from experimental thrombosis but also exhibit a marked defect in haemostasis, which may reflect the well-documented role of PLCγ2 signalling downstream of GPIbα and $\alpha_{IIb}\beta_3$ (Mangin et al. 2003; Rathore et al. 2004; Wang et al. 2000). Several other scaffolding and effector proteins assemble into the signalosome, including the GTP exchange factor Vav1 and 3 (Pearce et al. 2004), the small GTPase Rac1 (Pleines et al. 2009; Stefanini et al. 2012) and signal transducer and activator of transcription 3 (STAT3) (Zhou et al. 2013). All the proteins in the signalosome do not carry the same weight in the signalling cascade. While Syk (Poole et al. 1997), SLP76 and to a lesser degree LAT (Judd et al. 2002; Bezman et al. 2008) are critical for the activation of PLCγ2, a functional redundancy between closely related proteins such as SFK, Btk and Tec, Vav1 and 3 limits the consequence of their isolated deficiency (Table 1).

Activation of PLCγ2 leads to hydrolysis of its substrate, phosphatidylinositol-4,5-bisphosphate (PIP2), to form the two second messengers, inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), that respectively trigger the release of Ca^{2+} from intracellular stores and activation of protein kinase C (PKC). At this level, the signalling pathways downstream ITAM receptors, including GPVI, and GPCR converge and act synergistically. The guanine nucleotide exchange factor, CalDAG-GEFI, senses increased levels of cytosolic calcium and facilitates the rapid but reversible activation of the small GTPase Rap1 (Stefanini et al. 2009). Sustained activation of Rap1 results from PKC activation downstream of GPVI and feedback activation through the G_i-coupled receptor for ADP, $P2Y_{12}$ (Stefanini et al. 2012). Therefore, GPVI signalling depends on the ability of the CalDAG-GEFI/Rap1 couple to respond to threshold levels of Ca^{2+} by inducing ADP release via the activation of a Rac1, ADP that in turn triggers the second wave of PKC/Rap1 activation. Rap1 activation promotes integrin activation, thromboxane A2 generation and granule secretion, and the importance of this pathway is demonstrated by the fact that a deficiency in CalDAG-GEFI protects mice from collagen-induced thrombosis (Crittenden et al. 2004).

GPVI Ligands

Since, the initial observation that platelets lacking GPVI do not respond to collagen, numerous publications have provided clear evidence that GPVI is a central platelet receptor for collagens (Clemetson and Clemetson 2001; Nieswandt and Watson 2003). GPVI binds to the fibrillar and non-fibrillar forms of collagen, but not to soluble forms (Jung et al. 2008). Synthetic triple-helical collagen-like peptides (CRP) have been developed and are particularly convenient for the study of GPVI (Morton et al. 1995; Pasquet et al. 1999). GPVI binding increases with GPO content, but this minimal motif is not sufficient to secure GPVI binding, suggesting that nearby additional primary sequences must be involved (Smethurst et al. 2007; Jarvis et al. 2008).

Several snake venom peptides were reported to mediate their effects on activating platelets through GPVI. Some of them were extensively utilized as selective agonists, representing useful tools to study the function of GPVI (Andrews et al. 2001). This is notably the case of convulxin, a C-type lectin, which was of importance for GPVI cloning (Polgar et al. 1997; Jandrot-Perrus et al. 1997). Other snake venom toxins, which are more or less specific for GPVI, comprise alborhagin, alboaggregin A and trymucitin (Andrews et al. 2001).

In the last decade, a series of publications highlighted a multiplicity of additional GPVI natural ligands, notably adhesive proteins. First, GPVI was reported to directly bind to laminin, thereby promoting platelet activation and spreading (Inoue et al. 2006). This observation was confirmed independently in a study suggesting that the vascular α5-chain, but not α4-chain laminins promote platelet activation through GPVI (Schaff et al. 2013). In addition, Bultmann and collaborators proposed GPVI as a potential receptor for fibronectin that might participate in platelet/endothelial cell interaction, thereby promoting atheroprogression (Bultmann et al. 2010). The authors provided evidence of a direct GPVI/fibronectin interaction in a purified system and showed that soluble GPVI-Fc and a blocking anti-GPVI antibody reduced platelet adhesion to immobilized fibronectin under flow conditions. These observations were recently challenged in a study, which could not confirm a major role of GPVI as a platelet receptor for fibronectin (Maurer et al. 2015). Indeed, the absence or blockade of GPVI did not impair platelet adhesion and activation onto plasma or cellular fibronectin immobilized under their soluble or fibrillar forms. Recently, two independent groups reported a novel role for GPVI as a functional receptor for fibrin (Mammadova-Bach et al. 2015; Alshehri et al. 2015a). Both publications provided evidence that GPVI supports platelet adhesion under low and elevated blood flow conditions, resulting in activation, aggregation and procoagulant activity of platelets, suggesting a novel role for GPVI in enhancing thrombus growth. Beside adhesive proteins, GPVI was proposed to interact with a surface receptor of the IgG family, EMMPRIN, which could thereby mediate platelet/monocyte interactions and allow the recruitment of these aggregates at the vessel wall (Seizer et al. 2009; Schulz et al. 2011). GPVI has also been proposed to bind to globular adiponectin, an adipocyte-derived cytokine, which could promote platelet activation and aggregation (Riba et al. 2008). This observation will need to be confirmed and its physiological relevance identified. Finally,

it has recently been described that GPVI binds to charged hydrophobic ligands including diesel exhaust particles and large polysaccharides, thereby activating platelets (Alshehri et al. 2015b). In summary, even though GPVI is still mainly considered as a platelet receptor for collagen, the increasing number of additional natural ligands identified raises questions about the function of GPVI and the physiological relevance of these interactions. Whereas it becomes evident that the pathophysiological role of GPVI is not limited to arterial thrombosis, identification of specific binding sites for its ligands and development of selective blockers are mandatory to improve our knowledge on this receptor.

GPVI in Platelet Physiology

GPVI expression is restricted to platelets and megakaryocytes (Jandrot-Perrus et al. 2000). GPVI expression starts early and is upregulated at the late stage of megakaryocyte differentiation at which it is functional (Berlanga et al. 2000; Lagrue-Lak-Hal et al. 2001), but in contrast to α2β1 does not appear to regulate proplatelet formation (Sabri et al. 2004).

Using flow cytometry, a density of 3730 ± 453 GPVI copies was determined on human platelets with a 1.5-fold variation between individuals (Best et al. 2003). In contrast, quantitative proteomic analysis estimated the number of GPVI copies per platelet at 9600 (Burkhart et al. 2012). The reason of this important difference is not elucidated but could be linked to an internal pool of GPVI that has been detected by transmission electron microscopy (Suzuki et al. 2003). This pool was localized on the membranes of the surface-connected open canalicular system and α-granules and observed to be redistributed to the surface membrane and to microparticles following platelet activation, but requires to be quantified (Table 1).

The GP6 locus is highly polymorphic and two common haplotypes are associated with differences in protein expression resulting in distinct levels of platelet responses to specific ligand (Joutsi-Korhonen et al. 2003; Watkins et al. 2006). In fact, in cells expressing recombinant GPVI in variable amounts, collagen-triggered responses were dependent on receptor density (Chen et al. 2002). Interestingly, however, in vitro thrombus formation was not different with the blood of mice expressing 20 % or 100 % GPVI (Best et al. 2003) suggesting that moderate variations in GPVI expression should not represent a significant risk factor for thrombosis.

The function of GPVI is subject to two types of control resulting from homotypic interactions on one hand and from proteolysis on the other. Miura and co-workers, using soluble recombinant GPVI either dimeric (GPVI-Fc) or monomeric, demonstrated that only GPVI dimers bind to collagen with a good affinity consistent with structural analysis (Miura et al. 2002). Studies, using antibodies that distinguish between monomers and dimers, showed that platelet GPVI exists in the two forms but that GPVI dimerization is an active process closely regulated and which promotes binding to collagen (Jung et al. 2009; Loyau et al. 2012). GPVI dimerization is prevented by NO and prostacyclin continuously produced by endothelial cells in healthy vessels, but it is facilitated by platelet stimulation by shear-induced von Willebrand interaction with GPIb-V-IX, ADP or thrombin (Loyau et al. 2012). It is thus assumed that in pathological conditions, GPVI dimerization would increase the efficiency of platelet adhesion and activation onto collagen. An additional step is reached with the clustering of the receptor into higher ordered multimers by its ligands, a step required to initiate signalling. Convulxin, and to a lesser extent collagen, were reported to trigger the rapid formation of high molecular weight complexes composed disulphide-linked GPVI (Arthur et al. 2007b) involving Cys 338 within the cytoplasmic domain. However, this Cys is missing in mouse GPVI and whether disulphide-linked GPVI complexes are relevant in thrombosis remains to be elucidated.

Proteolysis of the extracellular domain of GPVI produces two fragments: a 55 kDa soluble fragment (sGPVI) representing almost the entire extracellular domain of GPVI and a 10 kDa membrane-associated remnant. The shedding of GPVI is induced by activation-dependent and independent pathways. Prolonged exposure to GPVI ligands (collagen, convulxin, CRP) triggers an activation-dependent shedding of GPVI resulting from its cleavage by several metalloproteinases including ADAM17 and ADAM10 (Bender et al. 2010). The dissociation of calmodulin from its binding motif in the cytoplasmic domain of GPVI leads to the activation proteases of the ADAM family (Gardiner et al. 2004). Both elevated shear and the coagulation factor Xa were reported to induce GPVI shedding by ADAM10-dependent and activation-independent mechanisms (Al-Tamimi et al. 2011, 2012b). Anti-GPVI antibodies have been shown to induce depletion of platelet surface GPVI in vivo and, for some but not all, in vitro. The mechanisms responsible for antibody-induced GPVI depletion are multiple, including internalization and shedding, and appear to be dependent on the antibody and the context (Rabie et al. 2007). As examples some antibodies, but not all, act via the engagement of platelet FcγRIIa, and some but not all also result in thrombocytopenia (Boylan et al. 2004; Arthur et al. 2007a; Nurden et al. 2009). High levels of sGPVI have been reported in the plasma of patients with (1) coronary artery stenosis (Al-Tamimi et al. 2012b), (2) disseminated intravascular coagulation (Al-Tamimi et al. 2011), and (3) autoimmune anti-GPVI antibodies (Gardiner et al. 2008). Plasma levels of sGPVI are thus increasingly considered as being a reliable

marker of in vivo platelet activation (Al-Tamimi et al. 2012a).

The first recognized function of GPVI is to be a receptor for fibrillar collagens (mainly of types I and III). The heterologous expression of GPVI is sufficient to induce cell adhesion to collagen (Jandrot-Perrus et al. 2000). However, it is accepted that initial recruitment of platelets is ensured by the GPIb-V-IX interaction with collagen-bound VWF, especially under elevated blood flow conditions. This interaction induces GPVI dimerization that promotes firm binding to collagen (Loyau et al. 2012). GPVI is required for stable platelet adhesion, through direct interaction with collagen but also indirectly through its ability to promote sustained platelet activation, thereby upregulating platelet integrins (Nieswandt et al. 2001b; Nieswandt and Watson 2003). Yet, synergy between GPVI and other platelet receptors including GPIb-V-IX, integrin $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ enables to achieve optimal rates and extents of thrombus formation in flowing blood (Lecut et al. 2004b; Siljander et al. 2004; Pugh et al. 2010). Moreover, GPVI clustering by collagen triggers the secretion of thromboxane A2 (TXA2) and ADP, two key soluble agonists that in turn promote effective thrombus growth and stability (Nieswandt et al. 2001a).

Importantly, GPVI plays a central role in collagen-induced exposure of procoagulant phospholipids at the platelet surface, which permits the assembly of the coagulation complexes tennase and prothrombinase (Heemskerk et al. 1999; Lecut et al. 2003). The resulting efficient local thrombin generation at the site of collagen-activated platelets is assumed to be critical for the formation of the thrombus core composed of hyperactivated and stacked platelets (Ivanciu et al. 2014; Stalker et al. 2014). Furthermore, thrombin triggers the formation of fibrin and it was recently shown that GPVI is also a receptor of polymerized fibrin (Alshehri et al. 2015a; Mammadova-Bach et al. 2015). The GPVI-fibrin interaction allows the recruitment of additional platelets and further amplifies thrombin generation and thus promotes thrombus stability and growth (Mammadova-Bach et al. 2015).

The Role of GPVI in Thrombus Formation and Stability

The absence or blockade of GPVI in whole blood perfused under large range of wall shear rate conditions over immobilized collagen has been widely shown to abolish thrombus formation with only few residual platelets adhering to the surface (Nieswandt et al. 2001b). Whereas this assay is widely accepted as an "in vitro thrombosis model," concluding from these observations that GPVI is key in thrombosis is probably overrated given that this model relies primarily on collagen. Of note, Cheli et al. reported that perfusion of whole blood from GPVI-deficient mice resulted in the formation of normal thrombi on a fibroblast matrix, indicating that additional platelet receptors can promote thrombus growth (Cheli et al. 2008). This study highlights the major impact of the choice of the adhesive surface on the experimental outcome. To convincingly address the role of GPVI in thrombus formation in vitro, a seductive and relevant strategy was based on the use of atherosclerotic plaque homogenates. Such an approach has been used by the group of Wolfgang Siess, which showed a major role for GPVI in in vitro thrombus formation (Penz et al. 2005). This key role for GPVI is in agreement with the high content of type I collagen fibres contained in atheroma plaques. The role of GPVI in thrombus formation has also been extensively studied in vivo. Contrasting data arise from various thrombosis models performed on healthy mouse vessels, with studies showing a crucial importance of GPVI in platelet recruitment and thrombus growth (Massberg et al. 2003), whereas other reported a minor role (Mangin et al. 2006). These discrepancies probably relate on the model and experimental conditions utilized which might expose different amounts of GPVI ligands, notably collagen. They could also find an explanation in compensatory mechanisms including thrombin generation at site of injury, which could compensate for the lack of GPVI deficiency. The role of GPVI in arterial thrombosis became much clearer in models closer to pathological conditions based on atherosclerotic plaque rupture in $ApoE^{-/-}$ mice. Kuijpers and co-workers showed that GPVI deficiency significantly impaired thrombus formation caused by ultrasound-induced plaque rupture (Kuijpers et al. 2009). This result was confirmed by an independent group, which also showed that GPVI is critical in a model of mechanical plaque rupture with a needle (Hechler and Gachet 2011).

GPVI is believed to participate in the initiation of thrombus formation through its ability to interact with several exposed subendothelial proteins, mainly collagen. These interactions initiate a signalling cascade leading to sustained integrin $\alpha_{IIb}\beta_3$ activation, which in turn supports platelet recruitment, i.e. thrombus growth (Nieswandt and Watson 2003). The reason why GPVI promotes a signal strong enough to support platelet aggregation, while other adhesive receptors do not, probably relies in its unique ability to efficiently release the potent soluble agonists, ADP and TxA2. Whereas GPVI has been proposed to initiate thrombus formation, it has been reported that the thrombosis defect in some animal models were not in the initial phase of thrombus growth but rather in the phase of thrombus stabilization as evidenced by a marked embolization in GPVI-deficient mice (Bender et al. 2011). This observation might be the consequence of the defective activation and release of soluble agonists occurring in GPVI-deficient

platelets, which ultimately results in a decrease in $\alpha_{IIb}\beta_3$ activation and thrombus instability. Alternatively, this observation could also suggest that GPVI directly participates in thrombus growth by supporting platelet/platelet interaction. This has recently be proposed in a study showing that blockade of GPVI reduces platelet recruitment to a growing thrombus, potentially through interaction via fibrin (Mammadova-Bach et al. 2015). Moreover, GPVI also appears to regulate thrombus growth, i.e. platelet/platelet interaction independently of fibrin (Maurer et al. 2015). Further studies are required to identify the molecular mechanism involved in this process.

A role for GPVI in ischemic stroke has also been evidenced in mice immunodepleted for this receptor in a model of transient middle cerebral artery occlusion (Kleinschnitz et al. 2007). GPVI-deficient mice presented a significant decrease in infarct volume with no apparent bleeding complications. The effect of GPVI in this process has been proposed to rely on a thrombo-inflammatory role of this receptor rather than a classical haemostatic function (Nieswandt et al. 2011).

GPVI in Haemostasis

Overall, the reported clinical observations of patients suffering of a GPVI deficiency indicate that the symptoms are rather benign going from no manifestation to easy bruising epistaxis and gum bleeding (Matus et al. 2013; Dumont et al. 2009). In fact, due to the paucity of the symptoms, it is likely that GPVI deficiencies are most often undiagnosed. However, more severe symptoms have occasionally been reported (Hermans et al. 2009) suggesting that the association of a GPVI deficiency with other factors that may affect the platelet count or functions, or the haemostatic function of the endothelium could lead to more severe haemorrhagic manifestations.

Five mutations in the GP6 gene have so far been described: two are insertions in exons 4 (Dumont et al. 2009) and 6 (Matus et al. 2013), one is a deletion in exon 3 (Hermans et al. 2009), all leading to reading frame shift and a premature stop codon; two others are missense mutations (R38C and S175N) altering the structure and the expression of the protein (Dumont et al. 2009; Hermans et al. 2009). Transmission was recessive with patients being homozygous (four patients) or compound heterozygous (two patients). Acquired GPVI deficiencies are more frequently reported. Most often these are patients with autoimmune thrombocytopenia characterized by the presence of autoantibodies that cause GPVI shedding and/or internalization (Arthur et al. 2007a). Consistent with the observations in patients, the genetic or antibody-induced absence of GPVI in mice has no serious haemorrhagic consequence.

The scarcity of bleeding in patients suffering from a GPVI deficiency, even severe, shows that GPVI is not strictly required to ensure physiological haemostasis, which is permitted thanks to the redundancy of the activation pathways in platelets (Kato et al. 2003; Lockyer et al. 2006). In particular, the vasoconstriction in response to vascular injury at the site of the lesion facilitates the GPIb/vWF interaction and platelet recruitment at the site of the lesion. This also encourages the development of new antithrombotic strategies based on inhibiting GPVI function (Dutting et al. 2012; Zahid et al. 2012).

GPVI in Inflammation

In addition to being the cellular orchestrators of thrombus formation, it is now evident that platelets are also major actors of inflammatory reactions (Fig. 2). In particular, it is well established that platelets promote leukocyte infiltration in inflamed tissues and organs. Although it may seem counter-intuitive, as platelets are mostly known for securing blood vessels and maintaining their barrier function, platelets have also been shown to promote the opening of endothelial junctions and thereby oedema formation, one of the earliest steps of inflammatory reactions (Boilard et al. 2012; Hillgruber et al. 2015; Hara et al. 2010; Petri et al. 2010). Interactions of platelets with the inflamed vasculature and local release of soluble compounds from activated platelets constitute the predominant basic mechanism by which platelets enhance vascular permeability and leukocyte infiltration. The identity of the involved platelet receptors is highly dependent on the inflammatory context, which is defined by the cause and site of inflammation, but it appears that there are situations in which GPVI plays a central role (Gros et al. 2014). In particular, GPVI was shown to mediate the recruitment of platelets to the inflamed joints in a mouse model of autoimmune rheumatoid arthritis (Boilard et al. 2012; Cloutier et al. 2012). In this model, platelets were responsible for the increased permeability of the synovial microvasculature and for the local recruitment of neutrophils. These pro-inflammatory effects of platelets were proposed to be dependent on GPVI, whose engagement led to the release of serotonin and microparticles in synovial fluid that, in turn, promoted gap formation in joint vasculature and the secretion of the neutrophil chemoattractant interleukin-8 by synovial fibroblasts, thus amplifying murine inflammatory arthritis. Remarkably, synovial fluid from patients with rheumatoid arthritis also displays elevated levels of serotonin and platelet-derived microparticles. Therefore, GPVI-dependent platelet activation could contribute to these increases and might represent a promising

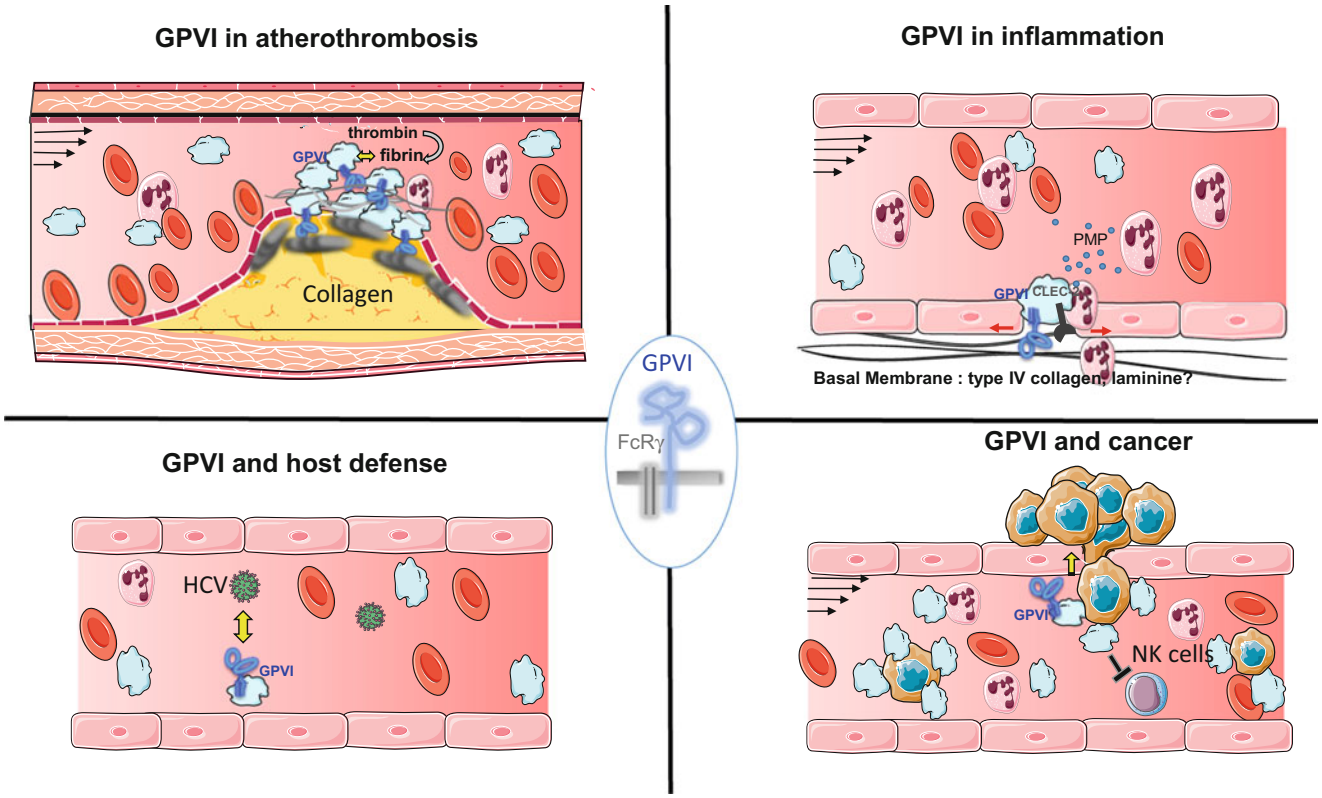

Fig. 2 Schematic representation of GPVI functions in thrombosis and beyond. These schemes illustrate the roles of GPVI in thrombus formation, inflammation, interaction with pathogens and cancer dissemination as described in the text. *PMP* platelet microparticle, *HCV* hepatitis C virus

therapeutic target to dampen inflammation in autoimmune rheumatoid arthritis.

Another inflammatory situation in which GPVI is suspected to play an important role is during glomerulonephritis. Clinical observations have suggested that platelets could mediate or amplify glomerular inflammation in various forms of glomerulonephritis (Zoja and Remuzzi 1995). Like in rheumatoid arthritis, platelets increased vascular permeability and leukocyte infiltration in the inflamed glomerulus, thus promoting glomerular injury and dysfunction. Clinical studies launched years ago to determine the possible efficacy of antiplatelet agents in progressive renal disease and using inhibitors of ADP-mediated platelet activation were mostly inconclusive. Instead, they underscored the need to identify the exact mechanisms by which platelets intervene in glomerular disease. Insights regarding these mechanisms have been brought by more recent studies using a mouse model of immune complex-mediated glomerulonephritis. In this model, GPVI was shown to support the early adhesion of platelets to the inflamed glomerular vasculature and subsequent platelet P-selectin-dependent leukocyte infiltration (Devi et al. 2010; Kuligowski et al. 2006). These findings are thus consistent with a mechanism whereby GPVI plays a critical role in the initiation of leukocyte-dependent glomerular injury, a process that is fundamental to the pathogenesis of many forms of glomerulonephritis.

Adhesion and migration within tissues is not the only leukocyte function that platelets can modulate. In fact, platelets are also able to regulate various effector functions of leukocytes including phagocytosis, generation of reactive oxygen species, granule secretion, cytokine production, as well as the release of neutrophil extracellular traps (Gros et al. 2014). How platelets regulate all these leukocyte functions remains unclear, but there is experimental evidence indicating that GPVI contributes to certain regulatory effects of platelets. For instance, a role for GPVI in the regulation of MMP-9 production by innate immune cells was suggested by the results showing that platelets stimulated MMP-9 production in monocytes in the presence of collagen (Galt et al. 2001). Concordantly, GPVI-dependent enhancement of MMP-9 secretion by neutrophils was recently reported in a mouse model of immune complex-mediated inflammation (Gros et al. 2015). Therefore, platelet GPVI might be considered as a collagen sensor used by leukocytes, so that they can release collagenases where appropriate. However, it appears that GPVI does not solely act as a bridge between collagen, platelets and leukocytes. In fact, besides collagen and other extracellular ligands like fibrin (Alshehri et al. 2015a; Mammadova-Bach

et al. 2015), it has been shown that GPVI is also capable of supporting direct platelet/leukocyte interactions and reciprocal regulations. As mentioned above, EMMPRIN, a surface receptor widely expressed in human tumours but also by platelets, monocytes, and activated lymphocytes, was recently identified as a ligand for GPVI (Seizer et al. 2009; Schulz et al. 2011). Furthermore, microspheres coated with a chimeric form of GPVI consisting of its extracellular domain fused to the Fc part of a human IgG1 molecule (Jandrot-Perrus et al. 2000) were shown to bind to neutrophils accumulating in inflamed microvessels during immune complex-mediated inflammation (Gros et al. 2015). Given the ability of EMMPRIN to stimulate the production of various MMPs, including MMP-9, one could postulate that EMMPRIN mediates the GPVI-dependent induction of MMP-9 production and secretion in monocytes and neutrophils.

While platelets promote oedema formation and leukocyte infiltration and activities, all events potentially damaging for blood vessels, recent studies have shown that in parallel, platelets continuously secure the inflamed vasculature by preventing bleeding throughout the entire course of inflammation (Goerge et al. 2008; Ho-Tin-Noe et al. 2009). This protective action of platelets was reported in various mouse models of inflammation including immune complex-mediated and irritant contact dermatitis (Goerge et al. 2008), glomerulonephritis (Hirahashi et al. 2009), endotoxin-induced acute lung injury (Goerge et al. 2008; Boulaftali et al. 2013) and solid tumours (Ho-Tin-Noe et al. 2008). Together with CLEC-2, another ITAM-associated platelet receptor, GPVI was identified as being responsible for this beneficial effect of platelets in at least two of these situations, immune complex-mediated dermatitis and endotoxin-induced acute lung injury (Boulaftali et al. 2013). In contrast, in these models, prevention of inflammatory bleeding was shown to be independent of the classical mechanisms of haemostasis (Goerge et al. 2008; Boulaftali et al. 2013). In the context of inflammation, prevention of bleeding by platelets and GPVI is thus mostly independent of thrombus formation. The results from a recent study indicate that instead, GPVI mediates the recruitment of individual platelets to seal vascular breaches due to neutrophil trafficking through inflamed post-capillary venules (Gros et al. 2015). Considering that GPVI is the main platelet receptor for collagen, it is likely that single platelets stop bleeding by covering small areas where the basement membrane gets exposed and disrupted by neutrophils. Nevertheless, whereas single adherent platelets could exert a purely mechanical action by plugging small holes in the endothelial lining, it was shown that defective ITAM signalling, either due to a genetic deficiency or to pharmacological inhibition, partially impaired the ability of platelets to prevent inflammatory bleeding (Boulaftali et al. 2013; Gros et al. 2015). Thus, in addition to GPVI-dependent platelet adhesion, GPVI and/or CLEC-2 signal-mediated platelet responses likely also contribute to sealing of the vascular breaches inflicted by neutrophils. In that regard, it was shown that even when not causing aggregation, collagen binding to GPVI can lead to the release of soluble platelet factors such as angiopoietin-1, serotonin, and ATP, all of which are regulators of endothelial cell barrier function (Ollivier et al. 2014).

Finally, the implication of GPVI in various inflammatory functions of platelets, as well as its ability to mediate the release of potent immunomodulators and regulators of vascular permeability without evoking platelet aggregation or procoagulant activity, provides a new perspective on GPVI that somehow reconciles it with its immune receptor nature.

Avenues for Future

Development of new efficient and safe anti-thrombotic drugs

If we take into account the important role that GPVI may play in atherothrombosis and little effect of its deficit on the physiological haemostasis, targeting GPVI appears as an attractive strategy to treat thrombosis without bleeding side effects. Research activities are mainly focus on two strategies: antibody-based inhibition of GPVI and competitive inhibition of GPVI binding to collagen. The later approach is based on the use of soluble dimeric recombinant protein (GPVI-Fc) that binds to collagen, purified or in atherosclerotic plaques (Jandrot-Perrus et al. 2000; Bultmann et al. 2010). Infusion of GPVI-Fc was observed to reduce thrombus formation following arterial injury without increasing bleeding in different animal models (Massberg et al. 2004; Jamasbi et al. 2015). GPVI-Fc (Revacept) was found as safe and well tolerated in a phase I study (Ungerer et al. 2011) and is under safety and feasibility evaluation in a phase II study in patients with symptomatic carotid artery disease (clinicaltrials.gov/NCT01645306). The former strategy is based on the use of antibodies. Several blocking antibodies were reported to inhibit thrombosis in animal models (Li et al. 2007; Mangin et al. 2012; Jamasbi et al. 2015) without increasing the bleeding time. Monovalent Fab fragments are preferable to avoid GPVI dimerization and cross-linking to FcγRIIA on human platelets and need to be humanized (Muzard et al. 2009). A transgenic mouse, humanized for GPVI, has been obtained allowing preclinical studies (Mangin et al. 2012). Phase I clinical trials are awaited to ensure the safety of these antibodies, but the fact that patients with a genetic and total deficiency or autoantibodies do not bleed, allows to be confident in the results.

GPVI and immune defence

In invertebrate and early vertebrates, one cell type, the haemocytes, mediates host defence and haemostasis. It is hypothesized that during evolution, the haemostatic and host defence functions diverged as specialized cell types support them. However, platelets have retained structures and functions of immune cells and still contribute to host defence. Since they are equipped with multiple receptors, platelets sense their environment and answer to danger signal: principally endothelial damages but also inflammatory signals as described above and even pathogens. Platelets interact with parasites, bacteria and viruses (Morrell 2014; Cox et al. 2011; Assinger 2014). In this area, Hepatitis C virus was found to bind to the extracellular domain of GPVI by a mechanism that could mimic collagen binding (Zahn et al. 2006). As a consequence of the receptor cross-linking, platelet P-selectin exposure may lead to subsequent removal of platelets from the circulation by the reticuloendothelial system. The recent observation that GPVI interacts with fibrin raises the possibility that fibrin(ogen)-bound pathogens may indirectly activate platelets via GPVI. Another unsolved issue is whether GPVI activation leads to the secretion of antimicrobial factors.

GPVI and cancer

Tumour cells interact with components of the haemostatic system, including platelets. The contribution of platelets and platelet activation to malignancy progression is complex and bidirectional and may underlie cancer growth and dissemination. Regarding the role of GPVI, the data so far reported showed that in GPVI-deficient mice, experimental metastasis based on inoculation of lung carcinoma and melanoma was reduced (Jain et al. 2009). Interestingly, there is growing evidence that platelets may facilitate tumour immune escape and metastasis, at least in part by modulating NK cell reactivity (Placke et al. 2011), and the place of GPVI in this process merits examination. The possible contribution of GPVI to the well-described pro-malignancy effects of tumour cell-associated EMMPRIN (Yan et al. 2005) is raised.

Take-Home Messages

GPVI is

- Involved in the initiation and growth of the thrombus
- Critically involved in atherothrombosis but dispensable for physiological haemostasis
- Contributing to the patrol role of platelets with respect to inflammation and host defence
- Contributing to the platelet-cancer cells dialogue
- A seducing target to develop new drugs associating safety to efficacy towards thrombosis and even beyond

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The Role of CLEC-2 in and Beyond the Vasculature

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Abstract

C-type lectin-like receptor 2 (CLEC-2) is a glycoprotein expressed on the surface of platelets that induces platelet activation including aggregation. Binding of the endogenous ligand podoplanin or the exogenous ligand rhodocytin to CLEC-2 induces tyrosine phosphorylation of the hemITAM motif expressed in its cytoplasmic tail. This hemITAM phosphorylation induces subsequent downstream signalling leading to calcium mobilisation and platelet activation. Although CLEC-2 stimulation induces powerful platelet activation, the interaction of CLEC-2 with podoplanin is not crucial for haemostasis. During development, CLEC-2 interacts with podoplanin on lymphatic endothelial cells (LECs) and is crucial for blood/lymphatic vessels separation, as well as development of the cerebrovasculature. Moreover, CLEC-2 critically maintains the integrity of high-endothelial venules within lymph nodes post-development through its interaction with podoplanin on fibroblastic reticular cells.

Under pathologic conditions, blockade of the CLEC-2/podoplanin interaction has been shown to elicit both detrimental and beneficial effects in a disease-dependent manner. The interaction has been shown to be detrimental in the case of tumour metastasis, HIV propagation and salmonella-mediated liver thrombosis and inflammation. In contrast, upregulation of podoplanin on TH17 cells impairs T-cell expansion and survival in multiple sclerosis and results in enhanced resolution of the disease. This book chapter will discuss the structure and function of the novel platelet receptor CLEC-2 from its discovery to the latest studies carried out on the physiological and pathological role of CLEC-2. This chapter is divided into four sections that cover the identification of CLEC-2 on platelets, the key signalling pathways involved, the role of CLEC-2 in embryonic development and its function in and beyond the vasculature.

Introduction

In 2000, Colonna et al. performed a bioinformatics study and identified the CLEC-2 gene *Clec1B* on chromosome 12 in the Dectin-1 gene cluster. The CLEC-2 gene is located near the genes for six other C-type lectin receptors and was identified as a novel member of the C-type lectin-like superfamily (Colonna et al. 2000). Originally, CLEC-2 mRNA was found in the liver and on some haematopoietic cells, in particular dendritic cells (DCs) and natural killer cells in human and mice (Colonna et al. 2000). Later, in 2006, CLEC-2 mRNA and CLEC-2 protein expression was found to be abundant in the megakaryocyte/platelet lineage, liver sinusoidal endothelial cells and liver Kupffer cells, although expression in liver has yet to be independently confirmed (Chaipan et al. 2006; Suzuki-Inoue et al. 2006; Senis et al.

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2007). In 2009, CLEC-2 was further shown to be expressed on subsets of murine myeloid cells and dendritic cells under basal conditions as well as on a variety of leukocyte subsets in response to inflammatory stimuli (Kerrigan et al. 2009; Acton et al. 2012). However, a recent study identified a much more restrictive expression profile, demonstrating CLEC-2- expression to be localised on platelets and on a subset of circulating inflammatory dendritic cells, which was lost upon entry into secondary lymphoid organs (Lowe et al. 2015a, b). The discrepancy in regard to expression to haematopoietic subsets is explained by off-target actions of the available antibodies. Investigation of the function of CLEC-2 in platelets was facilitated by its identification as a receptor for the snake venom rhodocytin using affinity chromatography and mass spectrometry (Suzuki-Inoue et al. 2006). CLEC-2 is a type II membrane protein with an extracellular carbohydrate-like recognition domain, a transmembrane domain and a cytoplasmic tail that is essential for downstream signalling. The CLEC-2 cytosolic tail has a hemi (or half) immunoreceptor tyrosine-based activation motif (ITAM) which consists of a single YXXL sequence which is phosphorylated by Src and Syk tyrosine kinases. CLEC-2 has two well-characterised ligands: an exogenous ligand, rhodocytin, and an endogenous ligand, podoplanin. In addition, it has been shown to be activated by the sulphated sugar, fucoidan, which also activates the platelet collagen receptor GPVI.

Podoplanin is a transmembrane glycoprotein that is widely expressed outside of the vasculature under physiological conditions including on kidney podocytes, alveolar type 1 epithelial cells, lymphatic endothelial cells (LECs), cerebral choroid plexus and many epithelial beds as well as being induced in subsets of haematopoietic cells. The expression of podoplanin on LECs is crucial for prevention of mixing of the blood and lymphatic vasculatures during development through its interaction with platelet CLEC-2- (Bertozzi et al. 2010; Herzog et al. 2013; Lowe et al. 2015a, b). On kidney podocytes, the absence of podoplanin was suggested to impair blood filtration, resulting in the presence of proteins in the urine (Koop et al. 2008). However, with the generation of transgenic mice with targeted deletion of podoplanin on kidney podocytes, the function of podoplanin in the kidney, both physiologically and under inflammatory conditions, is now questionable and requires further investigation (Rayes and Watson, unpublished). Podoplanin has been shown to be upregulated under pathologic conditions, such as inflammation, cancer and autoimmune disease on inflammatory macrophages, tumour cells and T-helper 17 (Th17) cells, respectively, and in development.

The role of podoplanin and CLEC-2 has been studied in a variety of disorders. It is important to note that the beneficial or detrimental effect of the CLEC-2-podoplanin interaction seems to be disease-dependent. Upregulation of podoplanin on macrophages and tumour cells induces platelet aggregation and thrombosis, whereas the expression of podoplanin on TH17 cells alters TH17 cell function and survival and improves recovery in a mouse model of multiple sclerosis (spontaneous experimental autoimmune encephalomyelitis) on a susceptible background.

In this chapter, CLEC-2 signalling pathways and the role of CLEC-2 in developmental pathways and in both thrombotic and non-thrombotic disorders will be discussed.

CLEC-2 Signalling

(Hem)ITAM Receptors in Platelets

As mentioned above, the C-type lectin receptor CLEC-2 is a glycoprotein receptor and a member of the (hem)ITAM family of receptors, which includes other receptors such as the B-cell antigen receptor, the low affinity immune receptor FcγRIIa and the major platelet signalling collagen receptor, GPVI/FcRγ chain (referred to as GPVI). An ITAM consists of two YXXL sequences (single amino acid code) separated by 6–12 amino acids. HemITAM receptors such as CLEC-2 differ as they contain only one YXXL sequence in their intracellular tails (Fuller et al. 2007). CLEC-2 signalling is additionally dependent on a conserved triacidic amino acid sequence immediately upstream of the hemITAM motif, which is also a feature of all but one of the other five known hemITAM-containing receptors (Hughes et al. 2013; Ruckrich and Steinle 2013). CLEC-2 is expressed as a dimer, thus providing two YXXL sequences (Fig. 1).

(Hem)ITAM Signalling

Upon receptor engagement via endogenous or exogenous ligands, the tyrosine residues contained within ITAM domains become targets of phosphorylation via Src family kinases (SFKs) such as Src and Lyn (Senis et al. 2014). A key difference between CLEC-2 and traditional ITAM signalling is that the hemITAM motif appears to be more reliant upon spleen tyrosine kinase (Syk) than SFKs for initial phosphorylation, with SFKs being important for activation of Syk and for signalling further downstream (Spalton et al. 2009; Severin et al. 2011). Upon platelet activation by agonists such as rhodocytin or podoplanin, the hemITAM sequence is phosphorylated, allowing recruitment and docking of the tandem SH2 domain-containing kinase Syk. Once recruited to the phosphorylated hemITAM via its SH2 domains, Syk undergoes a conformational change, moving from an auto-inhibited to an active conformation. In this active conformation, Syk undergoes further auto- and trans-phosphorylation. The latter is mediated by SFKs, allowing for full activation of Syk and phosphorylation of

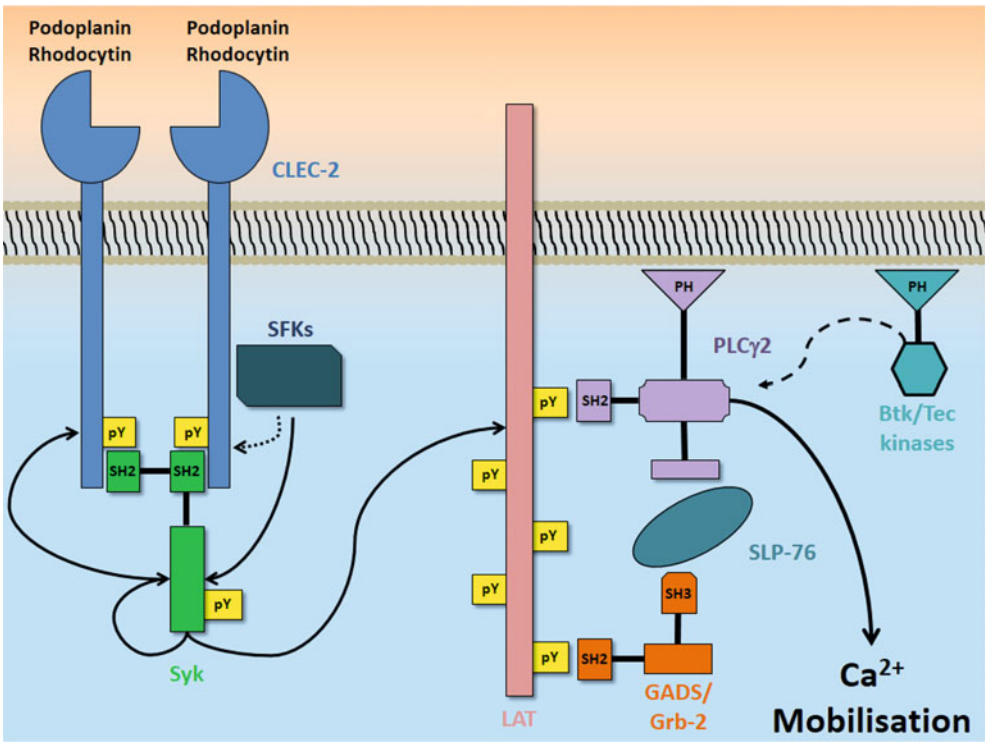

Fig. 1 Graphical representation of the proximal events in CLEC-2-mediated platelet activation. Upon receptor engagement, the hemITAM contained within the intracellular tail of CLEC-2 becomes phosphorylated by Syk and possibly also by Src family kinases. HemITAM phosphorylation allows recruitment of Syk via its SH2 domains, allowing downstream phosphorylation of LAT and the formation of the LAT signalosome. The result of this signalosome formation is the mobilisation of intracellular Ca^{2+} via recruitment and activation of PLCγ2

several downstream proteins. One of the most important of these is LAT, an adapter protein containing several tyrosine residues that undergo phosphorylation. LAT has no intrinsic activity and serves as the core of the 'LAT signalosome', allowing the docking and activation of many of the effectors of this pathway including Grb-2, Gads, SLP-76, PI3-K, Btk and PLCγ2 (Suzuki-Inoue et al. 2006; Fuller et al. 2007). PLCγ2 is generally considered to be the most important, giving rise to formation of inositol 1,4,5-trisphosphate (IP3) and 1,2-dacylglycerol (DAG) which mobilise Ca^{2+} and activate protein kinase C, respectively. These changes culminate in the exocytosis of intracellular granules, such as α- and dense granules, as well as inside-out activation of platelet integrins including $\alpha_{IIb}\beta_3$ (Hughes et al. 2015; Zheng et al. 2015). The release of ADP from dense granules and formation of thromboxane A_2, made *de novo* from membrane phospholipids, play critical positive feedback roles in activation of CLEC-2 in human platelets (Pollitt et al. 2010). CLEC-2 activation in human is also dependent on the Rho GTPase, Rac (Pollitt et al. 2010). This contrasts with activation by the collagen ITAM receptor GPVI which shows a reduced rather than complete dependency on the role of the two feedback messengers and Rac.

Functional Roles of CLEC-2 Signalling

Platelet activation and aggregation is a multistep process that involves different receptors and signalling pathways with the aim of prevention of excessive blood loss following vessel injury. CLEC-2 is expressed on platelets with an average copy number of 2000 receptor per platelet. Several studies have investigated a possible role of CLEC-2 in haemostasis and thrombus formation using various mouse models. CLEC-2-deficient platelets aggregate normally in response to classical platelet agonists but not to rhodocytin. Moreover, CLEC-2-deficient platelets have normal adhesion and spreading on extracellular matrices including collagen, laminin, von Willebrand factor and fibrinogen (Suzuki-Inoue et al. 2010). Several groups have investigated the consequence of CLEC-2 deficiency in a tail bleeding assay (haemostasis) or laser, photochemical or $FeCl_3$ thrombosis model with contrasting results. Using an anti-CLEC-2 antibody to deplete CLEC-2 from the platelet surface in vivo, May et al. (2009) showed a prolonged bleeding time and profound defect in arterial thrombus formation following $FeCl_3$ injury (May et al. 2009). Using irradiated mice reconstituted with Clec-$2^{-/-}$ foetal liver, Suzuki-Inoue

et al. (2010) showed a reduction in thrombus formation following photochemical injury but no significant change in tail bleeding times (Suzuki-Inoue et al. 2010). In contrast, Hughes et al. (2010) did not observe a significant change in tail bleeding time or aggregation on collagen at high shear using radiation chimeric mice reconstituted with CLEC-2-deficient bone marrow, and additionally were unable to demonstrate phosphorylation of CLEC-2 under flow conditions, providing indirect evidence that it is not activated (Hughes et al. 2010). The reason for the discrepancy between the various studies remains unclear. A recent study showed a profound defect in thrombus formation in mice double deficient in GPVI and CLEC-2 suggesting possible compensatory roles of the two (hem)ITAM receptors (Bender et al. 2013), although this which must involve adhesion and signalling as a similar result is not seen in mice deficient in Syk. Overall the consensus is that CLEC-2 has a minor role (at best) in haemostasis, consistent with the absence of an identified ligand in the vasculature, and that its primary role is to support many of the non-canonical roles for platelets that are beginning to emerge.

CLEC-2 in Development

Blood-Lymphatic Vascular Separation Defect in CLEC-2-Deficient Mice

Two decades ago, mice deficient in the downstream CLEC-2 signalling molecule, Syk, were shown to develop subcutaneous haemorrhages in the skin and oedema at mid-gestation (Cheng et al. 1995; Turner et al. 1995). A similar phenotype was observed in mice deficient in the adaptor protein SLP-76 (Clements et al. 1998; Pivniouk et al. 1998; Clements et al. 1999) or PLC-γ2 (Wang et al. 2000). The development of haemorrhages and oedema was later shown to result from aberrant connections between the blood and lymphatic vasculatures causing the appearance of blood-filled lymphatic vessels and impaired lymphatic drainage, respectively (Abtahian et al. 2003; Ichise et al. 2009). Support for the involvement of CLEC-2 in this phenotype came following the characterisation of mice deficient in its endogenous ligand, podoplanin, which were also shown to harbour blood-filled lymphatic vessels in the skin and gut at birth (Schacht et al. 2003), a phenotype later identified in CLEC-2-deficient embryos in mid-gestation (Bertozzi et al. 2010; Suzuki-Inoue et al. 2010; Finney et al. 2012). Both CLEC-2- and podoplanin-deficient mice experience almost 100 % perinatal mortality that is thought to be caused by defective lymphatic function and fluid retention leading to a failure to inflate the lungs (Schacht et al. 2003; Finney et al. 2012). Furthermore, the mice that survived for sufficient time to suckle developed chylous ascites, further indicative of impaired lymphatic function (Abtahian et al. 2003; Finney et al. 2012; Hess et al. 2014).

Identifying the Causative CLEC-2-Expressing Cell That Underlies the Defects in Lymphatic Development

The mechanism by which podoplanin and CLEC-2 prevent the mixing of the blood and lymphatic vasculatures during development remains controversial. A role for podoplanin specifically on the lymphatic endothelium has been confirmed by the presence of blood-filled lymphatic vessels in mice with a Tie-2-Cre-mediated deletion of podoplanin or the glycosyltransferase enzyme, T-synthase, which is important for the glycosylation of the podoplanin extracellular domain (Fu et al. 2008; Herzog et al. 2013). The interacting CLEC-2-expressing cell type has been investigated through a series of lineage tracing studies that initially pinpointed Syk/SLP-76 activity in a circulating endothelial progenitor (Abtahian et al. 2003; Sebzda et al. 2006) but which was later shown to be a circulating cell of the myeloid lineage (Bohmer et al. 2010). Using a series of transgenic mice, a role for Syk in macrophages, T- and B-lymphocytes, was eliminated, while the loss of CLEC-2, Syk or SLP-76 in the megakaryocyte/platelet lineage (generated by crossing floxed homozygotes to a PF4-Cre transgenic) was shown to be indispensable for blood-lymphatic vascular separation (Bertozzi et al. 2010; Finney et al. 2012).

A role for CLEC-2 on platelets/megakaryocytes is supported by the presence of blood-filled lymphatic vessels following the selective ablation of this lineage using diphtheria toxin (again achieved using a PF4-Cre transgenic), through treatment with aspirin *in utero* or in mice that lack functional megakaryocytes due to a deficiency in the transcription factor Meis-1 (Carramolino et al. 2010; Uhrin et al. 2010). Furthermore, the contribution of platelet-specific effects over megakaryocytes is supported by the development of blood-filled intestinal lymphatic vessels in irradiated mice reconstituted with Syk-, SLP-76-, PLCγ2- or CLEC-2-deficient bone marrow, where the number of circulating megakaryocytes is negligible (Ichise et al. 2009; Bertozzi et al. 2010; Finney et al. 2012).

Unravelling the Molecular Mechanism of the Impairment in Lymphatic Development

Initially it was proposed that a direct interaction between platelets and LECs at the cardinal vein was critical to ‘clot off’ the primary budding lymphatic structures and prevent

the formation of blood-lymphatic vascular connections (Bertozzi et al. 2010; Uhrin et al. 2010; Hess et al. 2014). However, high-resolution microscopy studies of developing embryos have failed to identify any connection between the blood and lymphatic vasculatures at this stage (Hagerling et al. 2013). Furthermore, blood-filled lymphatic vessels can be seen in irradiated adult mice reconstituted with CLEC-2- or Syk-deficient bone marrow where the lymphatic system is already established (Bertozzi et al. 2010; Finney et al. 2012). It was recently proposed that a thrombus, formed through podoplanin-induced CLEC-2 activation on platelets, forms at the joining of the thoracic duct and the subclavian vein to prevent backflow of blood into the lymphatic system during development and throughout adulthood (Hess et al. 2014). However, an important role for thrombus formation is disputed by the absence of a blood-lymphatic vascular separation defect in mice lacking the major platelet integrin $\alpha_{IIb}\beta_3$ (Uhrin et al. 2010).

It has also been proposed that one or more of the bioactive molecules or growth factors that are abundant in platelet dense, α- and lysosomal secretory granules and released upon their activation could contribute towards influencing lymphatic endothelial cell behaviour. So far, there has been little evidence to support this mechanism since patients or mice with defective dense- or α-granule secretion syndromes known as Hermansky-Pudlak syndrome or grey platelet syndrome, respectively, have not been described to exhibit lymphatic defects (e.g. Deppermann et al. 2013). Platelets additionally store large concentrations of the bioactive lipid sphingosine-1-phosphate (S1-P), which have been shown to have potent effects on endothelial cell migration, but while the loss of S1-P in mice is associated with widespread haemorrhaging and embryonic lethality, no phenotype of blood-lymphatic mixing was observed (Liu et al. 2000).

Other potential mechanisms include the direct interaction between platelet-CLEC-2 and podoplanin on LECs, which has been shown to inhibit LEC migration (Finney et al. 2012). It is proposed that these inhibitory signals are critical to arrest the growth of budding lymphatic vessels and prevent the formation of aberrant connections (anastomoses) with the blood vascular endothelium (Finney et al. 2012). The ability of CLEC-2 to cluster its receptor podoplanin on the surface of LECs was later demonstrated. This interaction supports platelet adhesion and is also proposed to inhibit signalling by podoplanin in LECs as shown in other cell types (Pollitt et al. 2014). The presence of anastomoses between veins and lymphatic veins has been described by many groups, including direct visualisation of mixing of the two vasculatures in mice with a platelet-specific loss of functional Syk (Hughes et al. 2015).

Other Developmental Functions of CLEC-2

Podoplanin was identified on fibroblastic reticular cells (FRCs) of the spleen over two decades ago and has since been shown to have several important roles in the organisation and function of lymphoid compartments (Farr et al. 1992; Yu et al. 2007). Recently, a number of these functions have been linked to CLEC-2 including trafficking of CLEC-2-expressing DCs to lymph nodes which is dependent on podoplanin expression on lymphatic endothelium and on FRCs (Acton et al. 2012). In addition, CLEC-2 has been shown to play a role in maintaining the integrity of high-endothelial venules within lymph nodes through its interaction with podoplanin on FRCs (Herzog et al. 2013). In this study, mice with a platelet/megakaryocyte-specific loss of CLEC-2 (i.e. PF4-Cre transgenic CLEC-$2^{fl/fl}$) developed blood-filled lymph nodes, and while this does not influence the primary immune response, it impairs the regulation of acquired immune responses (Herzog et al. 2013; Benezech et al. 2014). A much more severe phenotype is observed following constitutive loss of CLEC-2 or podoplanin where pups are born with a complete absence of mesenteric and inguinal lymph nodes due to a defect in lymph node maturation during late embryogenesis (Peters et al. 2011; Benezech et al. 2014). Significantly, lymph nodes are present in PF4-CLEC-2-deficient mice indicating that the developmental defect is not due to loss of platelet activation. This suggests a potential role for CLEC-2 outside of the platelet lineage during development and a second critical role for platelet-derived CLEC-2 in maintaining lymph node vascular integrity post-development.

A role for CLEC-2 in maintaining vascular integrity has been described in the developing brain. Podoplanin expression was shown to be restricted to the choroid plexus and ependymal lining of the ventricles at E16.5, but has since been seen to be widely expressed throughout the neuroepithelium between E10.5 and E12.5 (Williams et al. 1996; Schacht et al. 2003; Lowe et al. 2015a, b). Haemorrhages were first reported in the midbrain parenchyma of CLEC-2-deficient embryos at E12.0 (Tang et al. 2010). While severe cerebral haemorrhaging had been described in T-synthase-deficient embryos at E12.0, only recently were podoplanin-deficient embryos characterised and shown to exhibit extensive cerebral haemorrhaging between E11.5 and E12.5 (Lowe et al. 2015). Developing cerebral blood vessels in CLEC-2- and podoplanin-deficient mice were visibly tortuous and prone to haemorrhage at E10.5 caused by aberrant associations between the endothelium and the surrounding mural cells. CLEC-2-induced platelet aggregation is implicated in the haemorrhaging as bleeding is also seen in αIIb-deficient

embryos. Thus, activation of platelets by podoplanin-CLEC-2 leads to platelet aggregation and sealing of gaps in developing vessels. It remains unclear whether this also contributes to the altered patterning of vessels that is seen before the onset of haemorrhage.

CLEC-2- and podoplanin-deficient mice experience respiratory failure at birth due to the inability to inflate their lungs (Ramirez et al. 2003; Finney et al. 2012). This is likely secondary to impairment in lymphatic function leading to fluid retention. In addition, it has been speculated that this may also be due to loss of podoplanin on alveolar type I cells, although as yet there is no direct evidence for this (Millien et al. 2006).

Podoplanin-deficient mice have been reported to exhibit myocardial pathology, where hypoplasia in the myocardium is proposed to result from impaired development of the epicardium (Mahtab et al. 2008). However, no such phenotype has been reported in CLEC-2-deficient mice, suggesting podoplanin plays a functional role independent of CLEC-2 in cardiac development.

CLEC-2 Beyond Haemostasis: The Beneficial and Detrimental Role of the Interaction of CLEC-2 and Podoplanin

In the last few years, increasing evidence for a role of platelets in the regulation of inflammation, infection and cancer has emerged in human disease and mouse models. Platelets are now recognised as having multiple roles in inflammation and infection, including the ability to regulate leukocyte functions, such as secretion of granular contents and production of reactive oxygen species (ROS) from neutrophils (Gros et al. 2015), while the formation of a thrombus inside a blood vessel supports innate immunity through pathogen recognition and containment (Engelmann and Massberg 2013). Many

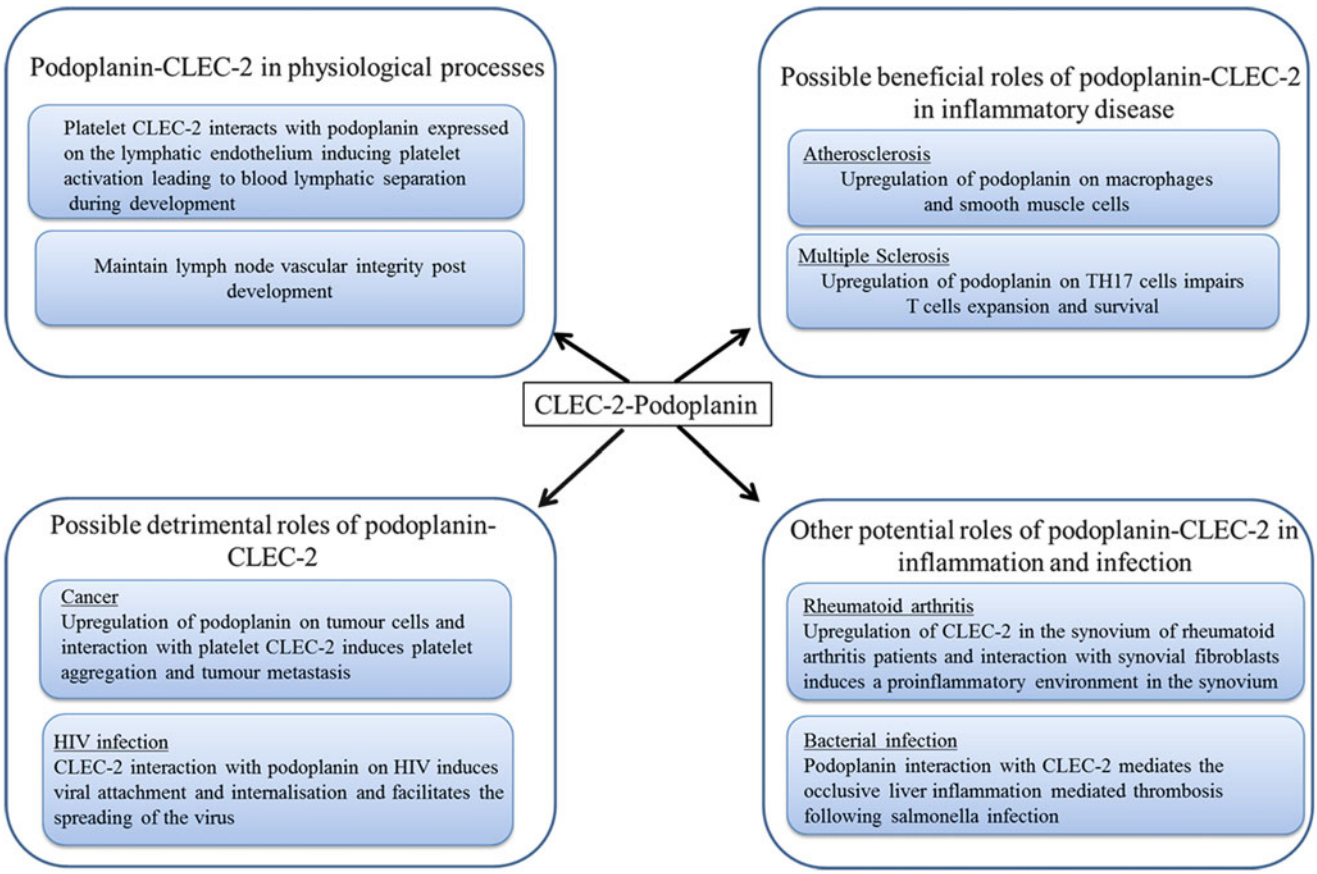

Fig. 2 Possible beneficial and detrimental roles of platelet CLEC-2/podoplanin interactions in normal physiology and in disease

of these new roles for platelets have been shown to involve CLEC-2 and podoplanin (Fig. 2).

The Role of CLEC-2-Podoplanin in Inflammatory Diseases

Multiple studies have shown the selective role of CLEC-2-podoplanin interaction in inflammatory and non-inflammatory conditions. The beneficial or detrimental effect depends largely on the inflammatory state and the nature of the platelet-leukocyte interaction. Indeed, in a mouse model of multiple sclerosis (MS), an autoimmune inflammatory disorder of the central nervous system (CNS), podoplanin expression on a subset of CD4+ cells, TH17 cells, alters TH17 expansion and survival resulting in an increased rate of resolution of the inflammation (Peters et al. 2015). This beneficial effect of podoplanin was abrogated by a global deletion of CLEC-2 showing that the interaction between CLEC-2 and podoplanin mediates the resolution of the inflammation. However, whether CLEC-2 on platelets, DCs or possibly B-cells (where expression is controversial) contributes to this effect remains unclear. Significantly, upregulation of podoplanin was also observed in human patients with active MS plaque but not in glioblastoma multiforme (GBM), an aggressive malignant primary brain tumour (Nylander et al. 2015) indicating this upregulation is disease-specific. Thus, podoplanin-CLEC-2 interaction seems to modulate the inflammatory response in MS infiltrates, and this effect depends on the presence of a specific microenvironment.

It has been suggested that platelets contribute to several stages of atherosclerosis, including initiation, lesion growth and thrombus formation upon plaque disruption. Interestingly, the level of expression of podoplanin on smooth muscle cells and macrophages in atherosclerotic lesions from human patients correlates with the severity of the lesion, although the significance of this remains to be determined (Hatakeyama et al. 2012).

CLEC-2 expression has been reported in synovium of patients with rheumatoid arthritis (RA), a chronic systemic inflammatory disease leading to progressive destruction of the articular cartilage and bone (Del Rey et al. 2014). Importantly, the interaction of synovial fibroblasts with platelet CLEC-2 has been shown to lead to an increase in the proinflammatory cytokines IL6 and IL8 (Del Rey et al. 2014). CLEC-2 expression on microvesicles is also increased in RA patients (Gitz et al. 2014). It has been speculated that the abundant levels of microvesicles in RA may induce potent pro-inflammatory effects on the synovial fluid (Boilard et al. 2010; Del Rey et al. 2014). Moreover, podoplanin expression is increased in areas of inflammation, and synovial fibroblast activation and synovial transformation (Ekwall et al. 2011; Miyamoto et al. 2013; Del Rey et al. 2014).

The Role of CLEC-2 and Podoplanin in Infection

There is increasing recognition of the importance of platelets in bacterial and viral infections (Cox et al. 2011; Chabert et al. 2015). Severe and sometimes life-threatening bleeding can occur in certain viral infections, with thrombocytopenia often arising from viral-induced platelet destruction, consumption or sequestration. Platelet-viral interactions are complex and will depend on the species of the virus and/or the platelet environment. One of the most important viral agents that CLEC-2 associates with is the human immunodeficiency virus (HIV-type 1). Zucker-Franklin et al. (1990) were the first group to visualise HIV internalisation by megakaryocytes and platelets (Zucker-Franklin et al. 1990). HIV-1-infected cells release HIV-1 virions expressing podoplanin on their capsules that allow HIV-1 to interact with CLEC-2 and DC-SIGN on platelets. This interaction mediates HIV-1 capture and facilitates the spread of infection (Chaipan et al. 2006; Chaipan et al. 2010). Encouragingly, a CLEC-2-specific antiserum reduced HIV-1 transmission by platelets by approximately 50 % (Chaipan et al. 2006). Podoplanin depletion only diminished CLEC-2-dependent HIV transmission in B-THP cells, a Raji B-cell line, suggesting that podoplanin incorporation for HIV spread is dependent on podoplanin expression in the targeted cell for HIV infection (Chaipan et al. 2010). Therefore, incorporation of a second, as yet unrecognised, CLEC-2 ligand may be responsible for CLEC-2-dependent capture of T-cell-derived and peripheral blood mononuclear cell-derived viruses, facilitating CLEC-2-driven HIV transmission (Ozaki et al. 2009; Chaipan et al. 2010; Lowe et al. 2015a, b).

In a mouse model of salmonella infection, Hitchcock et al. (2015) have recently reported that upregulation of podoplanin in the liver leads to an inflammation-driven occlusive thrombosis. Significantly, the venous thrombosis was substantially abrogated in the absence of CLEC-2 on platelets or in the absence of macrophages without affecting the time course of the bacteraemia. The upregulation of podoplanin in the liver is driven by a TLR4- and INF-γ-dependent inflammation. Given that CLEC-2 does not have a major role in haemostasis, this highlights the C-type lectin-like receptor as a novel target in some forms of thromboinflammatory disease.

The Role of CLEC-2 and Podoplanin in Cancer

A potential role of podoplanin in cancer metastasis has also emerged. Podoplanin is upregulated in a wide range of cancer types such as colorectal adenocarcinoma (Kato et al. 2003), testicular germ cell tumours (Kato et al. 2004), squamous cell carcinomas of the lung, cervix, oral cavity, larynx and mesothelioma and in tumours of the central nervous system (Kato et al. 2005; Kimura and

Kimura 2005; Schacht et al. 2005; Shibahara et al. 2006; Rodrigo et al. 2010). Tumour cells can interact with platelets to form heterogeneous aggregates which protect the invasive cells from shear stress and immunological assault (Gay and Felding-Habermann 2011). It is speculated that the coating of platelets allows the tumour cells to evade the immune system as well as supporting adherence to the vessel wall. The antihuman podoplanin antibody (MS-1 mAb) has been shown to reduce platelet aggregation in vitro and pulmonary metastasis in vivo (Takagi et al. 2013). This highlights podoplanin as a promising target for developing novel antitumour and anti-metastatic agents.

Take-Home Messages

- CLEC-2 interaction with podoplanin induces platelet activation and aggregation.
- During development, CLEC-2-podopanin interaction is crucial for blood/lymphatic vessel separation.
- Post-development, CLEC-2-podoplanin interaction maintains the integrity of high endothelial venules in lymph nodes.
- CLEC-2-podoplanin is not crucial for haemostasis.
- Detrimental role of CLEC-2-podoplanin: HIV propagation, salmonella-induced liver thrombosis and cancer metastasis.
- Beneficial role of CLEC-2-podoplanin inhibition of T-cell expansion and survival in multiple sclerosis.

Conclusion

Although CLEC-2 and podoplanin have been intensively studied in the last decade, many questions concerning their roles remain unanswered, most notably the roles outside of the vasculature. Anti-CLEC-2 or anti-podoplanin drugs may have a beneficial effect in many diseases, in particular in inflammatory disorders. Additional studies are required to fully understand the mechanism underlying the role of CLEC-2 and podoplanin in physiology and pathology.

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Anatomy of the Platelet Cytoskeleton

Hervé Falet

Abstract

The platelet cytoskeleton maintains the discoid shape of the resting platelet and mediates a rapid shape change in response to external stimuli at sites of vascular injury. At rest, specific cytoskeletal structures and molecules maintain the unique architecture of the platelet actin cytoskeleton, connect it to the plasma membrane, and prevent actin monomer assembly onto actin filaments. Platelet activation stimulates the interactions of cytoplasmic signals with actin regulatory proteins to initiate and amplify actin filament growth necessary for platelet shape change. This chapter reviews the cellular mechanisms and proteins maintaining the morphology of resting and activated platelets, focusing on the actin cytoskeleton and exciting recent mRNA and protein profiling studies, clinical observations, and mouse models.

Introduction

Following blood vessel injury and disruption of the vascular endothelium, platelets interact avidly with exposed elements of the basement membrane and rapidly change from resting discs to active forms, first by rounding and then by extending finger-like filopodia and spreading thin sheet-like lamellipodia. This shape change reaction is further amplified by soluble factors released by activated platelets. Shape change is orchestrated by the platelet cytoskeleton, which has therefore two main functions: (1) to maintain the biconvex discoid shape of the resting platelet and (2) to mediate a rapid shape change in response to external stimuli at sites of vascular injury. The platelet cytoskeleton is composed of actin and tubulin polymers and associated cytoskeletal proteins, which compose a large fraction of the platelet proteome. Mutations and single nucleotide polymorphisms (SNPs) in genes encoding cytoskeletal proteins often lead to altered platelet counts and mean platelet volume. Table 1 lists the major cytoskeletal proteins regulating the resting and activated platelet cytoskeleton, based on recent mRNA and protein profiling studies, clinical observations, and mouse models, while the major actin regulatory proteins regulating the platelet actin assembly reaction are listed in Table 2.

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The Cytoskeleton of the Resting Platelet

Actin

Actin is by far the most abundant protein in platelets, where it represents 15–20 % of the total cellular protein mass. Platelet actin is evenly distributed between β-actin and γ-actin, also called cytoplasmic actins 1 and 2, respectively (Rowley et al. 2011; Burkhart et al. 2012) (Table 1). In resting platelets, about 40 % of total actin is assembled into dynamic polymers or filaments (F-actin) that can be reversibly assembled from monomeric or globular actin (G-actin). The distribution of actin in filaments reaches 80 % in activated platelets.

Actin filaments are polarized structures, first recognized by electron microscopy by the way filaments can be decorated with the myosin head domain, which binds periodically along

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Table 1 Major cytoskeletal proteins regulating the resting and activated platelet cytoskeleton

	Human					Mouse		
Protein	Gene	Position	Monomers	mRNA	Mutation phenotype	Gene	mRNA	Mutation phenotype
Actin								
β-Actin, cytoplasmic 1	*ACTB*	7p22.1	795,000	2854.52		*Actb*	1607.58	
γ-Actin, cytoplasmic 2	*ACTG1*	17q25.3	791,000	654.78		*Actg1*	125.67	
Membrane cytoskeleton								
α1-Spectrin, erythroid	*SPTA1*	1q23.1	650	0.06		*Stpa1*	0.50	
α2-Spectrin, non-erythroid	*SPTAN1*	9q34.11	3200	1.40		*Sptan1*	18.40	
β_1-Spectrin, erythroid	*SPTB*	14q23.3	3900	21.95		*Sptb*	21.65	
β_2-Spectrin, non-erythroid	*SPTBN1*	2p16.2	4600	4.37		*Sptbn1*	63.23	
α-Adducin, erythroid	*ADD1*	4p16.3	3900	18.00		*Add1*	94.39	Mild thrombocytosis, small platelets (lack γ-adducin)
γ-Adducin	*ADD3*	10q25.1	2800	25.00		*Add3*	20.84	Normal
Tropomodulin 3	*TMOD3*	15q21.2	11,900	14.08		*Tmod3*	136.99	Impaired platelet production
Tropomyosin 4	*TPM4*	19p13.12	107,000	249.28	SNP-MPV	*Tpm4*	937.38	
Tropomyosin 1	*TPM1*	15q22.2	26,000	162.27	SNP-PLT	*Tpm1*	177.80	
Dematin	*DMTN*	8p21.1	14,500	308.43		*Dmtn*	0.15	Normal
Actin filament cross-linking and bundling proteins								
Filamin A	*FLNA*	Xq28	87,700	1086.03	Mild thrombocytopenia (in females), large platelets	*Flna*	867.89	Severe thrombocytopenia, large platelets
α-Actinin 1	*ACTN1*	14q24.1	92,100	512.41	Mild thrombocytopenia, large platelets	*Actn1*	312.98	
α-Actinin 4	*ACTN4*	19q13.2	45,600	24.12		*Actn4*	228.92	
Focal adhesion and membrane-associated proteins								
Talin 1	*TLN1*	9p13.3	116,000	909.67		*Tln1*	718.67	Impaired $\alpha_{IIb}\beta_3$ activation
Kindlin 3	*FERMT3*	11q13.1	116,000	696.36	Impaired $\alpha_{IIb}\beta_3$ activation	*Fermt3*	1374.70	Impaired $\alpha_{IIb}\beta_3$ activation
Vinculin	*VCL*	10q22.2	81,100	438.51		*Vcl*	75.40	Normal
Zyxin	*ZYX*	7q34	72,700	376.46		*Zyx*	2251.46	Normal
Paxillin	*PXN*	12q24.23	?	2.57		*Pxn*	1.24	Normal
Skelemin (myomesin 1)	*MYOM1*	18p11.31	?	4.33		*Myom1*	11.90	
Moesin	*MSN*	Xq12	34,800	141.45		*Msn*	109.42	Normal
Contractile proteins								
Myosin heavy chain IIA, non-muscle	*MYH9*	22q12.3	96,900	661.66	Mild thrombocytopenia, large platelets	*Myh9*	716.14	Severe thrombocytopenia, large platelets
LC20 (MRLC1)	*MYL9*	18p11.31	88,000	773.26		*Myl9*	7142.57	
LC17	*MYL6*	12q13.2	229,000	922.06		*Myl6*	1046.59	
Marginal band								
α1-Tubulin	*TUBA4A*	2q35	185,000	701.34		*Tuba1a*	294.17	
β_1-Tubulin, hematopoietic	*TUBB1*	20q13.3	144,000	2879.61	Severe thrombocytopenia, large platelets, SNP-MPV	*Tubb1*	3313.72	Mild thrombocytopenia, spherical platelets

Protein copy numbers (monomers) per platelet derive from Burkhart et al. (2012) Blood 120:e73–e82. mRNA transcripts (expressed as reads per kilobase of transcript per million mapped reads) derive from Rowley et al. (2011) Blood 118:e101–e111. *SNP-MPV* SNP associated with mean platelet volume, *SNP-PLT* SNP associated with blood platelet counts

Table 2 Major actin regulatory proteins regulating the platelet actin assembly reaction

Protein	Human					Mouse		
	Gene	Position	Monomers	mRNA	Mutation phenotype	Gene	mRNA	Mutation phenotype
Actin monomer-sequestering proteins								
Thymosin β_4	*TMSB4X*	Xq22.2	?	5313.27		*Tmsb4x*	53,308.40	
Profilin 1	*PFN1*	17q13.2	503,000	321.11		*Pfn1*	838.32	Mild thrombocytopenia, small platelets
Actin filament-capping proteins								
CapZ α1	*CAPZA1*	1p13.2	20,900	3.68		*Capza1*	5.56	
CapZ α2	*CAPZA2*	7q31.2	16,400	66.38		*Capza2*	75.34	
CapZ β	*CAPZB*	1p36.13	26,400	96.31		*Capzb*	277.26	
Gelsolin family								
Gelsolin	*GSN*	9q33.2	52,900	84.42		*Gsn*	550.34	Impaired actin assembly
Flightless 1	*FLII*	17p11.2	6800	30.84		*Flii*	31.84	
Supervillin	*SVIL*	10p11.23	620	7.79	SNP-MPV	*Svil*	2.31	Large platelets
ADF/cofilin family								
Cofilin 1, non-muscle	*CFL1*	11q13.1	244,000	339.56		*Cfl1*	853.24	Mild thrombocytopenia, large platelets
ADF (destrin)	*DSTN*	20p12.1	14,400	11.93		*Dstn*	146.96	Normal
Arp2/3 complex								
Arp2	*ACTR2*	2p14	30,300	23.09		*Actr2*	33.16	
Arp3	*ACTR3*	2q14.1	30,600	19.78		*Actr3*	21.26	
p41-Arc	*ARPC1B*	7q22.1	19,100	7.75		*Arpc1b*	133.87	
p34-Arc	*ARPC2*	2q35	17,400	84.63		*Arpc2*	389.84	
p21-Arc	*ARPC3*	12q24.11	27,500	17.18		*Arpc3*	299.13	
p20-Arc	*ARPC4*	3p25.3	26,000	168.27		*Arpc4*	40.87	
p16-Arc	*ARPC5*	1q25.3	22,900	149.37		*Arpc5*	90.31	
WASp family and actin nucleation-promoting factors								
WASp	*WAS*	Xp11.23	4000	4.94	Severe thrombocytopenia (in males), small platelets	*Was*	25.90	Mild thrombocytopenia
WIP	*WIPF1*	2q31.1	5400	552.88	Severe thrombocytopenia (platelets lack WASp)	*Wipf1*	75.26	Mild thrombocytopenia (platelets lack WASp)
N-WASP	*WASL*	7q31.32	?	0.61	SNP-PLT	*Wasl*	0.26	
WAVE2	*WASF2*	1p36.11	3100	13.45		*Wasf2*	16.45	Impaired platelet production
WAVE1	*WASF1*	6q21	1400	1.03		*Wasf1*	0.01	Normal
Cortactin	*CTTN*	11q13.3	10,800	234.57		*Cttn*	14.97	Normal
HS1	*HCLS1*	3q13.33	1600	4.75		*Hcls1*	47.34	Normal
Formins								
DIAPH1	*DIAPH1*	5q31.3	8100	92.77	Mild thrombocytopenia, large platelets	*Diaph1*	55.13	Normal
FHOD1	*FHOD1*	16q22.1	6600	3.53		*Fhod1*	6.47	
DAAM1	*DAAM1*	14q23.1	3800	7.84		*Daam1*	2.71	
INF2	*INF2*	14q32.33	7500	99.89		*Inf2*	162.86	

Protein copy numbers (monomers) per platelet derive from Burkhart et al. (2012) Blood 120:e73–e82. mRNA transcripts (expressed as reads per kilobase of transcript per million mapped reads) derive from Rowley et al. (2011) Blood 118:e101–e111. *SNP-MPV* SNP associated with mean platelet volume, *SNP-PLT* SNP associated with blood platelet counts

the filament length to define barbed and pointed ends. This polarity is reflected in different rates of monomer addition to the two ends. The barbed end of the filament is the preferred end of actin filament assembly in vitro, as it has high affinity for G-actin and elongates at a rate approximately ten times faster than the pointed end. Barbed ends are the only ends contributing to actin assembly in cells, particularly in platelets. When assembled, actin filaments can be cross-linked or bundled into higher-order structures or fragmented into smaller pieces. A large number of platelet cytoskeletal-associated proteins control these dynamic processes.

The Membrane Cytoskeleton

The morphology of the resting platelet is maintained by a membrane cytoskeleton, a thick protein meshwork composed principally of spectrin, adducin, actin filaments, and

actin-associated proteins laminating the cytoplasmic side of the platelet plasma membrane (Fig. 1) (Fox and Boyles 1988; Hartwig and DeSisto 1991). Analogies have been made with the well-characterized spectrin-based erythrocyte membrane cytoskeleton (Lux 2016). Platelet spectrin is composed of both erythroid and non-erythroid α/β-spectrin heterodimers forming 200-nm long tetramers assembled head-to-head. Assembly of spectrin into tetramers is required for platelet production by megakaryocytes (Patel-Hett et al. 2011). Contrary to erythrocytes, where spectrin is connected to short actin oligomers capped by adducin at their barbed end and tropomodulin at their pointed end (Lux 2016), platelet spectrin strands interconnect at the barbed ends of the actin filament network originating from the cytoplasm (Fox and Boyles 1988; Hartwig and DeSisto 1991). Therefore, the dense platelet spectrin lattice and its associated actin filaments assemble into a continuous ultrastructure.

Capture of actin filament barbed ends by spectrin requires adducin, which forms a high-affinity ternary complex with spectrin and actin (Fig. 1) (Gilligan et al. 2002; Barkalow et al. 2003). Platelet express both erythroid α-adducin (encoded by human *ADD1*, mouse *Add1*) and non-erythroid γ-adducin (human *ADD3*, mouse *Add3*). *Add1*-null mice lack both α- and γ-adducin in platelets and have slightly elevated platelet counts with reduced mean platelet volume (Robledo et al. 2008), indicating that α-adducin regulates both the expression of γ-adducin and the morphology of the resting platelet membrane cytoskeleton. By contrast, the role of γ-adducin appears redundant, as *Add3*-null mice have normal platelet counts and morphology and express α-adducin normally in platelets (Robledo et al. 2008).

Platelets express several other cytoskeletal proteins previously characterized in the erythrocyte membrane cytoskeleton. These include the pointed-end capping protein tropomodulin and tropomyosin, which binds along the side of the short actin oligomers of the erythrocyte membrane cytoskeleton, where it is believed to control their length (Lux 2016). Megakaryocytes and platelets principally express tropomodulin 3 (human *TMOD3*, mouse *Tmod3*), deficiency of which is embryonic lethal in mice (Sui et al. 2014). *Tmod3*-null embryos develop thrombocytopenia with large platelets, due to impaired platelet production by megakaryocytes, which show increased actin assembly and actin redistribution from the cortical membrane cytoskeleton to the cytoplasm (Sui et al. 2015). In humans, SNPs in *TPM1* and *TPM4* encoding tropomyosin 1 and 4 have been associated with altered platelet counts and mean platelet volume (Soranzo et al. 2009; Gieger et al. 2011; Qayyum et al. 2012). However, further functional studies are required to determine the role of tropomyosin 1 and 4 in megakaryocytes and platelets.

Dematin is an actin-bundling protein of the erythrocyte membrane skeleton abundantly expressed in platelets (Wieschhaus et al. 2012). Dematin consists of a C-terminal headpiece and an N-terminal tail that both contain F-actin binding sites. Dematin binds to spectrin and enhances its binding to actin filaments. Platelets lacking the dematin headpiece exhibit defects in calcium mobilization in response to multiple agonists, associated with concomitant inhibition of platelet aggregation and granule secretion, unveiling dematin as a regulator of internal calcium mobilization affecting multiple platelet cytoskeletal and signaling functions.

Cross-Linking and Bundling Proteins

Actin filaments in the resting platelet are organized into a rigid cytoplasmic scaffold by filamin A (FlnA; previously known as ABP-280), which cross-links actin filaments at a distinctive 90° angle, and by α-actinins, which tend to align actin filaments in parallel bundles.

FlnA (human *FLNA*, mouse *Flna*) is a large antiparallel homodimer that cross-links actin filaments, tethers membrane glycoproteins, and serves as a scaffold for signaling intermediates (Falet 2013). FlnA contains an N-terminal tandem calponin-homology actin-binding domain followed by 24 immunoglobulin-like repeats of 90–100 amino acid residues, the C-terminal of which mediates dimerization (Nakamura et al. 2007; Seo et al. 2009). Two calpain-sensitive hinges separate FlnA repeats into an elongated and linear rod 1 (repeats 1–15) that can bind to actin filaments independently of the actin-binding domain, a compact rod 2 (repeats 16–23), and the self-association domain (repeat 24) (Nakamura et al. 2007). Rod 2 contains most of the binding sites for FlnA partners, e.g., the von Willebrand factor (VWF) receptor subunit GPIbα (repeat 17) and β-integrins (repeat 21) (Nakamura et al. 2006; Kiema et al. 2006).

FlnA traverses the pores of the spectrin-based membrane cytoskeleton to link the VWF receptor GPIb-IX, specifically the GPIbα subunit (Cranmer et al. 2005, 2011; Nakamura et al. 2006), to the underlying actin filaments (Fig. 1). The FlnA–GPIbα linkage initiates in mature megakaryocytes and is constitutively maintained during the platelet lifespan (Begonja et al. 2011). It plays a critical role in maintaining the platelet discoid shape, as has been established in studies of Bernard–Soulier syndrome (BSS) platelets lacking the VWF receptor subunits GPIbα, GPIbβ, or GPIX, or in platelets lacking functional FlnA, all of which are abnormally large in size, are fragile, and circulate poorly. FlnA also interacts with the β-subunits of integrins, thereby competing with talin 1 (Kiema et al. 2006), an essential activator of integrin $\alpha_{IIb}\beta_3$ in activated platelets (Petrich et al. 2007; Nieswandt et al. 2007). FlnA binding to β-integrin subunits is not constitutive,

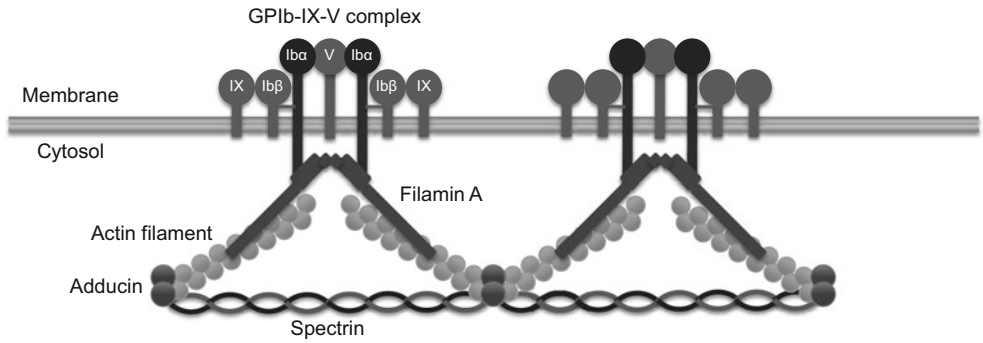

Fig. 1 Organization of the resting platelet cytoskeleton. Spectrin strands laminate the cytoplasmic surface of the plasma membrane and interconnect using barbed ends of actin filaments. Adducin binding to the actin filament barbed end targets spectrin. Actin filaments are cross-linked by FlnA, which provides the major membrane–cytoskeletal connection linking the actin cytoskeleton to the cytoplasmic tail of the GPIbα subunit of the VWF receptor complex GPIb-IX-V

but regulated by mechanotransduction (Ehrlicher et al. 2011), the significance of which for platelet production and function remains to be investigated.

The gene encoding FlnA is X-linked in both humans and mice. *FLNA* mutations causing early truncation of FlnA are typically embryonic lethal in males, due to cardiovascular defects and hemorrhage, and lead to the brain malformation disorder, periventricular heterotopia (PH) in females (Feng and Walsh 2004). Missense mutations cause otopalatodigital (OPD) syndrome spectrum disorders, primarily affecting skeletal development. Thrombocytopenia, bleeding tendency, and giant platelets have been associated with *FLNA* mutations and multiple FlnA degradation products have been observed in platelets of female carriers (Nurden et al. 2011; Berrou et al. 2013; Li et al. 2015). However, the distribution of mutant platelets is not easily detectable and analysis of their hemostatic function is cumbersome, due to the mosaicity of the platelet population and the high proportion of normal platelets in female carriers.

In mice, *Flna* deficiency is embryonic lethal in hemizygous males, due to pericardiac and visceral hemorrhage, severe cardiac structural defects, and aberrant vascular patterning (Hart et al. 2006; Feng et al. 2006). Heterozygous female mice carrier for *Flna* deficiency have mild thrombocytopenia, and more than 95 % of their platelets contain FlnA, which can be differentiated from *Flna*-null platelets by intracellular flow cytometry (Falet et al. 2010). Mice specifically lacking FlnA in the megakaryocyte/platelet lineage develop severe thrombocytopenia, giant platelets, and increased tail bleeding time, due to severely impaired platelet hemostatic functions (Falet et al. 2010). As expected, GPIbα is not linked to the actin cytoskeleton in *Flna*-null megakaryocytes and platelets, where its surface expression is reduced and altered (Falet et al. 2010; Begonja et al. 2011). *Flna*-null megakaryocytes prematurely release large and fragile platelets that undergo microvesiculation and are cleared rapidly from the circulation by macrophages (Begonja et al. 2011). These observations corroborate earlier studies showing that cleavage of FlnA by the protease calpain causes microvesiculation in aggregating platelets or during platelet storage (Robey et al. 1979; Basse et al. 1994). Thus, the FlnA–GPIbα linkage not only regulates platelet size but also maintains the mechanical stability of the platelet plasma membrane. *Flna*-null megakaryocytes have impaired demarcation membrane system (DMS) formation, which is likely due to FlnA interaction with the endocytic membrane-binding and membrane-deforming Fes/CIP4 homology Bin/amphiphysin/Rvs (F-BAR) protein PACSIN2, an internal component of the initiating DMS (Begonja et al. 2015). Expression of the FlnA paralog FlnB has been reported in megakaryocytes and platelets (Falet et al. 2010; Kanaji et al. 2012). However, FlnB does not appear to compensate for loss of FlnA.

The role of α-actinins in the actin organization of resting platelets is less well defined. α-Actinins belong to the spectrin gene superfamily, including α/β-spectrins and dystrophins. α-Actinins are antiparallel homodimers composed of an N-terminal tandem calponin-homology actin-binding domain, four spectrin repeats, and a C-terminal calmodulin-like domain. Platelets primarily express α-actinins 1 and 4. α-Actinin 1 is an abundant actin filament bundling protein often associated with adhesion sites, where it binds to the cytoplasmic tail of β_1-integrins, suggesting that α-actinin 1 links platelet actin filaments to the membrane, particularly at adhesion sites. Mutations in *ACTN1* encoding α-actinin 1 have been associated with mild thrombocytopenia, large platelets, and low risk for bleeding (Kunishima et al. 2013; Gueguen et al. 2013; Bottega et al. 2015; Yasutomi et al. 2015). These mutations in the α-actinin 1 actin-binding domain enhance association with actin filaments (Murphy et al. 2016). Mouse fetal liver cell-derived megakaryocytes transfected with α-actinin 1 variants display altered proplatelet formation and size (Kunishima et al. 2013).

Focal Adhesion and Membrane-Associated Proteins

Focal adhesions are integrin-associated actin-rich structures that enable cells to adhere to the extracellular matrix and at which protein complexes involved in signal transduction assemble. Platelet activation leads to a conformational change of integrin $\alpha_{IIb}\beta_3$ to promote fibrinogen binding and platelet aggregation. Several cytoskeletal proteins modulate integrin $\alpha_{IIb}\beta_3$ activation, including talin 1 (human *TLN1*, mouse *Tln1*) and kindlin 3 (human FERMT3, mouse *Fermt3*). Talin 1 is composed of an N-terminal head domain binding to β-integrin subunits, followed by an elongated C-terminal actin-binding rod domain. Talin 1 binding to β_3 is required for $\alpha_{IIb}\beta_3$ activation in platelets, as *Tln1*-null platelets have impaired agonist-mediated platelet aggregation (Petrich et al. 2007; Nieswandt et al. 2007). Mice specifically lacking talin 1 in platelets exhibit prolonged tail bleeding time and pathological gastrointestinal bleeding, similar to mice lacking the integrin β_3 (Hodivala-Dilke et al. 1999). *FERMT3* mutations cause leukocyte adhesion deficiency (LAD), which is characterized by impaired activation of the β_2-integrin in leukocytes and β_3 in platelets (Shattil et al. 2010; van de Vijver et al. 2012). *Fermt3*-null platelets exhibit defective integrin $\alpha_{IIb}\beta_3$ activation and impaired platelet aggregation (Moser et al. 2008). However, kindlins are unable to activate $\alpha_{IIb}\beta_3$ in the absence of talin 1 head domain (Ma et al. 2008; Harburger et al. 2009), suggesting that kindlins co-activate $\alpha_{IIb}\beta_3$ in synergy with talin 1.

Other notable platelet focal adhesion proteins include vinculin, zyxin, and paxillin. Vinculin (human *VCL*, mouse *Vcl*) links integrins to the actin cytoskeleton by virtue of binding of its N-terminal globular head domain to talin 1 and α-actinins, while its C-terminal tail region binds actin filaments, paxillin, and membrane phospholipids. Vinculin has been implicated in the transmission of mechanical forces from the extracellular matrix to the cytoskeleton of migrating cells (Bailly 2003). However, *Vcl*-null platelets display normal agonist-induced integrin $\alpha_{IIb}\beta_3$ activation, aggregation, spreading, and actin polymerization and organization and adhere normally to immobilized fibrinogen or collagen under both static and flow conditions (Mitsios et al. 2010), indicating that platelet vinculin is not required for the traditional functions of $\alpha_{IIb}\beta_3$ or the actin cytoskeleton. Both zyxin and paxillin contain lin-11/Islet-1/mec-3 (LIM) domains composed of zinc fingers that mediate protein–protein interactions and serve as targeting motif for focal adhesions. Similar to vinculin, the role of platelet zyxin and paxillin appears redundant based on the generation of mutant mice (Hoffman et al. 2003; Sakata et al. 2014).

Yeast two-hybrid system screening has identified skelemin (also called myomesin 1) as another cytoskeletal protein interacting with the integrin β_3 cytoplasmic tail (Reddy et al. 1998). Skelemin is a member of a superfamily of cytoskeletal proteins that regulate the three-dimensional organization of myosin filaments in skeletal muscle cells. Skelemin does not associate with $\alpha_{IIb}\beta_3$ in resting platelets but is recruited to $\alpha_{IIb}\beta_3$ during cell adhesion (Podolnikova et al. 2009). Biochemical and structural studies have shown that the skelemin-binding domain on β_3 is cryptic but becomes exposed as a result of $\alpha_{IIb}\beta_3$ binding to immobilized ligands (Podolnikova et al. 2009; Gorbatyuk et al. 2014). These studies illuminate a potential link between $\alpha_{IIb}\beta_3$ and actomyosin filaments, which may serve as a basis for generating the mechanical forces necessary for cell migration and remodeling.

Moesin is a member of the ezrin/radixin/moesin (ERM) family of proteins, which localize in cell extensions like filopodia and function as cross-linkers between the plasma membrane and actin filaments. Moesin associates with the tips of filopodia in spread platelets and associates with platelet/endothelial cell adhesion molecule 1 (PECAM-1) in lysates from thrombin-stimulated, but not resting platelets (Nakamura et al. 1995; Gamulescu et al. 2003), suggesting that moesin may play a role in platelet adhesion, linking PECAM-1 with the actin cytoskeleton. Mice lacking moesin develop normally and are fertile, with no obvious histological abnormalities. Particularly, targeted moesin deletion does not affect platelet aggregation in response to stimulation (Doi et al. 1999).

Contractile Proteins

Myosin-mediated contractile forces are critical for maintaining the integrity of a hemostatic plug at wound sites independently of thrombin and fibrin generation. The principal myosin isoform in platelets is non-muscle myosin heavy chain (NMMHC)-IIA (human *MYH9*, mouse *Myh9*). NMMHC-IIA is a parallel homodimer, whose two subunits are composed of an N-terminal head domain that binds actin filaments, a neck region, and a C-terminal coiled-coil rod domain holding the two chains together. A myosin regulatory light chain of 20 kDa (LC20, also called MRLC1) and an essential light chain of 17 kDa (LC17) bind each NMMHC-IIA subunit in the neck region, between the head and the tail. The functional properties of NMMHC-IIA are controlled by phosphorylation of both its heavy and light chains. Phosphorylated myosin assembles into filaments that are essential for its interaction with actin to form a contractile unit whose function is analogous to that of actomyosin filaments in smooth muscle cells. Phosphorylation of LC20 by myosin light chain kinase (MLCK), RhoA-associated kinase (ROCK), or p21-activated kinase 1 (PAK1) increases its actin-stimulated ATPase activity and is required for NMMHC-IIA to move along actin filaments, thereby

providing contractile force. LC17 is believed to contribute to the structural stability of NMMHC-IIA along with LC20. NMMHC-IIA is homogeneously distributed in resting platelets (Painter and Ginsberg 1984). Following activation, NMMHC-IIA localizes in the platelet center and contributes to the initiation of platelet shape change, platelet internal contraction, granule exocytosis, and centralization of GPIb-IX on the platelet surface (Kovacsovics and Hartwig 1996; Johnson et al. 2007).

MYH9 mutations cause MYH9-related disease (MYH9-RD), previously classified as May–Hegglin anomaly or Epstein, Fechtner, or Sebastian syndromes and characterized by macrothrombocytopenia, mild bleeding tendency, and leukocyte inclusions, with or without loss of hearing, cataract, or nephritis (Favier and Raslova 2015). The mechanisms of thrombocytopenia underlying *MYH9* mutations are linked to defects in proplatelet formation by megakaryocytes, which show decreased branching and increased tip size in cultures from patient progenitors (Pecci et al. 2010; Chen et al. 2013). In mice, NMMHC-IIA is required to form the megakaryocyte DMS and to maintain the megakaryocyte shape through internal tension and anchorage to the extracellular matrix (Eckly et al. 2009). Mice specifically lacking NMMHC-IIA in megakaryocytes develop severe macrothrombocytopenia. However, *Myh9* deletion in mouse megakaryocytes results in enhanced proplatelet formation (Chen et al. 2007; Leon et al. 2007; Eckly et al. 2009), suggesting that large *Myh9*-null platelets are prematurely released from the bone marrow and cleared rapidly from the circulation.

MYH10-encoded NMMHC-IIB is expressed in immature megakaryocytes, where it specifically localizes in the contractile ring (Lordier et al. 2012). NMMHC-IIB expression is silenced by the hematopoietic Runt-related transcription factor 1 (RUNX1), a requirement for megakaryocyte ploidization and maturation. Anomalous NMMHC-IIB expression in platelets has been proposed as a tool to identify inherited platelet disorders and myeloid neoplasms with abnormalities in RUNX1 and associated proteins (Antony-Debre et al. 2012). Mutations in RUNX1 have also been associated with reduced platelet expression of *MYL9*-encoded LC20 (Sun et al. 2007; Jalagadugula et al. 2010).

The Marginal Band

Microtubules contribute to the maintenance of the discoid shape of the resting platelet (Sadoul 2015). Microtubules are highly dynamic tubular polymers formed by the polymerization of a dimer of α- and β-tubulin. Approximately 40 % of tubulin in platelets is assembled into a specialized microtubule coil, called the marginal band, which lies at the periphery of resting platelets, below the plasma membrane. Many proteins bind microtubules, including the motor proteins kinesin and dynein, and other proteins important for regulating microtubule dynamics.

The marginal band is maintained in its resting state by both tubulin acetylation and antagonistic microtubule motors (Sadoul 2015). During platelet activation, microtubules undergo major reorganization, thereby contributing to the shape change of activated platelets. A dramatic tubulin deacetylation mediated by the cytoplasmic histone deacetylase 6 (HDAC6) occurs (Sadoul et al. 2012; Aslan et al. 2013). Dynein slides microtubules apart, resulting in marginal band extension and further coiling. New microtubule polymerization within the coiled marginal band leads to the formation of a smaller microtubule ring, in concerted action with NMMHC-IIA-mediated tension (Diagouraga et al. 2014). Microtubules are then reacetylated in spread platelets, and the capacity of HDAC6 to prevent tubulin hyperacetylation influences the speed of platelet spreading.

Microtubules play an essential role in proplatelet formation by megakaryocytes (Italiano et al. 1999; Patel et al. 2005; Bender et al. 2015b). The predominant β-tubulin isoform in platelets and megakaryocytes is the hematopoietic β_1-tubulin (human *TUBB1*, mouse *Tubb1*). Early studies have shown that *Tubb1*-null mice develop thrombocytopenia due to defective proplatelet production by megakaryocytes (Schwer et al. 2001). Circulating mouse *Tubb1*-null platelets have normal platelet size, but develop spherocytosis, lacking their characteristic discoid shape, and have defective marginal bands with reduced microtubule coilings and frequent kinks and breaks (Italiano et al. 2003). Following platelet activation, the disorganized microtubules in *Tubb1*-null platelets fail to condense into central rings and instead are dispersed in short bundles and linear arrays. More recently, an SNP in *TUBB1* has been associated with altered mean platelet volume in humans (Gieger et al. 2011), and *TUBB1* mutations have been associated with congenital macrothrombocytopenia due to impaired proplatelet formation by megakaryocytes (Kunishima et al. 2009, 2014; Stachele et al. 2015). The discrepancies between mouse and human phenotypes relative to platelet size and morphology are likely due to compensation by different β-tubulin isoforms.

The Cytoskeleton of the Activated Platelet

Actin Filament Assembly

Following receptor-mediated activation, platelet shape change proceeds through two recognizable steps. First, platelets convert from disc into an irregular or round shape. With further surface contact, platelets extend finger-like filopodia and spread thin sheet-like lamellipodia, as evidenced by electron microscopy (Fig. 2). Temporal and spatial actin filament assembly by specific cytoskeletal

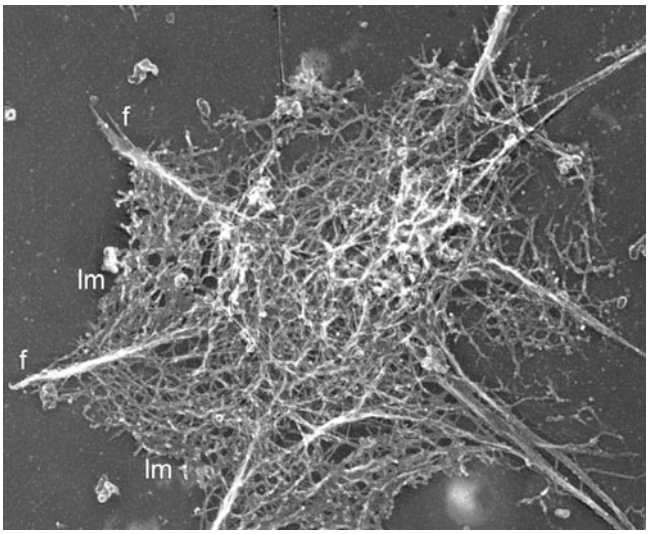

Fig. 2 Cytoskeleton of the activated platelet. Platelet activation is accompanied by a striking reorganization of the actin cytoskeleton. Platelets generate finger-like projections called filopodia (*f*) composed of long bundled actin filaments that derive from the cytoskeletal center. Platelets spread using thin sheet-like extensions called lamellipodia (*lm*) that are densely filled with a three-dimensional network of newly assembled short actin filaments

proteins orchestrates these morphological changes (Fig. 3 and Table 2) (Hartwig 1992).

In vitro, G-actin reversibly polymerizes into F-actin in three sequential phases defined as nucleation, elongation, and steady state. Nucleation is marked by a lag period in which G-actin slowly aggregates into short, unstable oligomers. Once a stable nucleus of three or four actin subunits is formed, it rapidly elongates into a filament by the addition of G-actin to its ends. In cells, actin monomers incorporated into filaments are bound to ATP. As filaments elongate, the bound ATP is slowly hydrolyzed to ADP. As a result of this hydrolysis, only actin filament barbed ends are composed of ATP-bound actin, and most of the filament consists of ADP-bound actin.

Actin Monomer-Sequestering Proteins and Actin Filament Capping Proteins

The G-actin–F-actin equilibrium of the resting platelet is principally maintained by two mechanisms that prohibit actin monomer addition to actin filaments (Fig. 3). First, actin subunits not incorporated into filaments complex with the actin monomer-sequestering proteins thymosin β_4 and profilin 1. Second, virtually all actin filament barbed ends are constitutively capped by the high-affinity actin filament capping protein CapZ (Barkalow et al. 1996). Therefore, actin assembly in platelets occurs when actin monomers release from thymosin β_4 and profilin 1 and add to uncapped actin filament barbed ends. Despite these control mechanisms, actin filaments are believed to dynamically turnover in resting platelets.

Thymosin β_4 (human *TMSB4X*, mouse *Tmsb4x*) and profilin 1 (human *PFN1*, mouse *Pfn1*) are two of the most abundant proteins in platelets. Thymosin β_4 forms a 1:1 complex with G-actin, in levels sufficient to account for essentially all sequestered actin monomers in platelets (Weber et al. 1992; Nachmias 1993). It has higher affinity for ATP-bound actin than for ADP-bound actin, suggesting that platelet thymosin β_4 sequesters primarily ATP-bound actin monomers ready to assemble into filaments. The proportion of G-actin bound to thymosin β_4 decreases following platelet activation (Nachmias et al. 1993). *Tmsb4x* deletion leads to partial embryonic lethality due to hemorrhage (Rossdeutsch et al. 2012). It is likely that other thymosins such as thymosin β_{10} compensate for thymosin β_4 loss in mice reaching adulthood. Megakaryocyte-specific *Pfn1* deletion in mice results in mild thrombocytopenia with small platelets due to premature release of platelets into the bone marrow and accelerated platelet clearance (Bender et al. 2014). However, actin assembly is minimally affected by profilin 1 deletion. Instead, *Pfn1*-null platelets contain hyper-stable and misarranged microtubules, revealing an unexpected novel function of profilin 1 as a regulator of microtubule organization.

CapZ (also called capping protein) is a key cellular component regulating actin filament assembly and organization, as it caps the barbed ends of actin filaments, preventing addition and loss of actin monomers. CapZ is a heterodimer composed of α- and β-subunits (Yamashita et al. 2003). The distribution of α-subunits varies among cell types, with platelets expressing both α1 and α2 (Nachmias et al. 1996; Hart et al. 1997). In platelets, the amount of CapZ bound to actin is proportional to that of F-actin (Barkalow et al. 1996). The kinetics of CapZ association with actin closely follows the kinetics of actin assembly and binding reaches a maximum when actin assembly ceases. The observations show that CapZ acts primarily as an actin assembly buffer to maintain actin filament barbed ends capped and inaccessible at rest and to terminate actin assembly in activated platelets by capturing exposed barbed ends. Because CapZ maintains most barbed ends capped in resting platelets (Barkalow et al. 1996), either filament ends must be uncapped, or barbed end nucleation sites must be generated de novo to trigger actin filament assembly. Both mechanisms contribute to actin filament assembly in activated platelets.

The Gelsolin Family

The actin filament severing and capping protein gelsolin (human *GSN*, mouse *Gsn*) is responsible for most of the

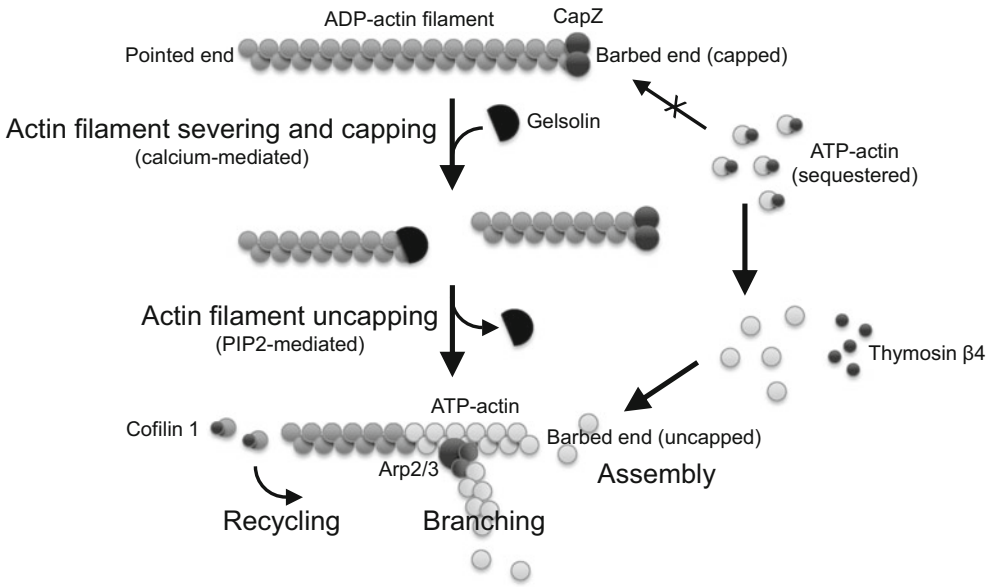

Fig. 3 Regulation of platelet actin assembly. In resting platelets, CapZ caps actin filament barbed ends, and thymosin β_4 sequesters ATP-bound actin monomers, thereby maintaining the large actin pool that drives actin filament assembly. Activation leads to rise of intracellular calcium and the activation of gelsolin. Gelsolin severs cortical actin filaments and caps the barbed ends, thereby inducing platelet rounding. PIP2 is robustly generated at the cytoplasmic surface of the platelet plasma membrane and inactivates gelsolin, thereby leading to the initiation of ATP-bound actin assembly onto exposed barbed ends and actin filament branching by the Arp2/3 complex. Actin filaments are subsequently buffered by CapZ, which recaps the barbed ends and terminates the assembly reaction. Subsequent actin filament treadmilling is stimulated by cofilin 1

actin assembly occurring during platelet activation (Witke et al. 1995; Barkalow et al. 1996). Gelsolin is one of the best-characterized molecules in terms of its effects on actin in vitro. It is activated to bind actin filaments in the presence of micromolar concentration of calcium. Association with actin begins with gelsolin 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 until membrane phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP2), mediate its dissociation (Janmey and Stossel 1987).

In resting platelets, the cytoplasmic calcium level is low, and gelsolin is not associated with actin. Following platelet activation, cytoplasmic calcium rises within seconds and activates gelsolin to bind and sever long cortical actin filaments into short ones (Fig. 2) (Lind et al. 1987). Gelsolin severing destabilizes the highly ordered and constrained structure formed between the membrane cytoskeleton and actin filaments cross-linked by FlnA, initiating platelet rounding, before the actin assembly reaction begins. Subsequently, the small guanosine triphosphatase (GTPase) Rac1 is activated and stimulates the synthesis of membrane PIP2 that mediate the dissociation of gelsolin from actin filament barbed ends, thereby promoting actin assembly (Hartwig et al. 1995; Azim et al. 2000; McCarty et al. 2005; Aslan and McCarty 2013). These new ATP-bound actin filaments are preferred for Arp2/3 complex-mediated actin nucleation and branching (Fig. 3) (Falet et al. 2002b). Platelets from *Gsn*-null mice have a severe reduction in their capacity to generate actin filament barbed ends, assemble actin, and spread following platelet activation (Witke et al. 1995; Barkalow et al. 1996; Falet et al. 2000, 2002b; Hoffmeister et al. 2001). *Gsn*-null mice have prolonged bleeding time (Witke et al. 1995), but normal platelet counts and size, indicating that gelsolin is dispensable for platelet production and survival. Gelsolin-mediated actin filament severing can be blocked by vasodilator-stimulated phosphoprotein (VASP) binding to the sides of actin filaments. VASP is a major substrate of protein kinase A in platelets, where its phosphorylation correlates with reduced activation. Consistently, mouse platelets lacking VASP have slightly increased adhesion and spreading (Hauser et al. 1999; Massberg et al. 2004).

Platelets express at least two other actin severing and capping proteins of the gelsolin family: flightless 1 (human *FLII*, mouse *Flii*) and supervillin (human *SVIL*, mouse *Svil*). In platelets, flightless 1 is present in a complex with leucine-rich repeat (in FLII) interacting protein 1 (LRRFIP1), an SNP of which has been associated with altered platelet functions (Goodall et al. 2010). An SNP in *SVIL* has been associated with low supervillin expression in platelets and increased platelet thrombus formation in the shear-dependent platelet function analyzer (PFA)-100 (Edelstein

et al. 2012). Consistent with these observations, *Svil*-null mouse platelets exhibit enhanced platelet thrombus formation at high shear stress, indicating that supervillin plays an inhibitory role in platelet adhesion and arterial thrombosis. The specific molecular details of flightless 1 and supervillin function in platelet actin assembly and remodeling have not yet been elucidated.

The ADF/Cofilin Family

Actin-depolymerizing factor (ADF)/cofilin is a family of actin-binding proteins that regulate both assembly and disassembly of actin filaments. Two distinct mechanisms have been proposed: severing and depolymerization. First, ADF/cofilin proteins can sever actin filaments to create barbed ends without capping, thereby promoting actin assembly. Second, ADF/cofilin proteins can depolymerize actin filaments at their pointed ends, thereby accelerating actin filament turnover in cells, a process called treadmilling (Carlier et al. 1997). ADF/cofilin proteins are primarily regulated by phosphorylation of serine 3 (Ser3), which inhibits their function.

Platelets express primarily non-muscle cofilin 1 (human *CFL1*, mouse *Cfl1*) and ADF (also called destrin; human *DSTN*, mouse *Dstn*). Cofilin 1 is phosphorylated on Ser3 in resting platelets and, therefore, is inactive (Davidson and Haslam 1994; Falet et al. 2005; Pandey et al. 2006). Following platelet activation, cofilin 1 is dephosphorylated and incorporates into the actin cytoskeleton, shortly after the peak of actin assembly occurs (Falet et al. 2005). Ser3 dephosphorylation and activation are maintained by $\alpha_{IIb}\beta_3$-mediated outside-in signals and are transient and reversible in the absence of integrin $\alpha_{IIb}\beta_3$ engagement, such as in platelets isolated from a patient with Glanzmann thrombasthenia expressing low integrin $\alpha_{IIb}\beta_3$ levels (Falet et al. 2005). The observations indicate that cofilin 1 is not essential for the initial polymerization of actin filaments that follows platelet activation, but for actin filament turnover mediated by outside-in signals (Fig. 3).

Cofilin 1-mediated actin filament turnover plays a critical role in the late stages of platelet production by megakaryocytes and in the proper sizing of platelets in the periphery, as mice specifically lacking cofilin 1 in the megakaryocyte lineage develop mild thrombocytopenia with large platelets due to defective platelet production (Bender et al. 2010). Macrothrombocytopenia is severe in mice expressing a germ line mutation in the cofilin 1 partner WD40 repeat-containing protein 1 (WDR1), the vertebrate homolog of actin-interacting protein 1 (AIP1) (Kile et al. 2007). Actin filament assembly and platelet spreading on immobilized fibrinogen are profoundly impaired in the absence of cofilin 1 (Bender et al. 2010). The observations show that cofilin 1 is essential for the maintenance of G-actin–F-actin equilibrium in resting platelets and for agonist-induced actin assembly. By contrast, *Dstn*-null mice lacking ADF have normal platelet counts, size, and actin assembly reaction following activation (Bender et al. 2010).

Actin Nucleation by the Arp2/3 Complex

The Arp2/3 complex plays a major role in the regulation of actin assembly in cells, as it nucleates actin filaments and localizes to the leading edge of crawling cells (Pollard and Borisy 2003). The Arp2/3 complex also initiates the actin-based motility of intracellular pathogenic bacteria such as *Shigella flexneri* and *Listeria monocytogenes*. The Arp2/3 complex is composed of two actin-related proteins, Arp2 and Arp3, and five additional subunits. Arp2 and Arp3 closely resemble the structure of G-actin and serve as nucleation sites for new actin filaments. In vitro, the Arp2/3 complex binds to the side of existing actin filaments to initiate the growth of a new filament at a 70° angle. These observations suggest that the Arp2/3 complex is responsible for generating a branched meshwork of actin filaments and de novo actin filament nucleation at the cell cortex that leads to cell movement. Binding of Arp2/3 complex to ATP-bound actin, as found at uncapped actin filament barbed ends, is preferred (Ichetovkin et al. 2002; Falet et al. 2002b). Therefore, the Arp2/3 complex does not explicitly initiate actin nucleation de novo, but rather amplifies actin filament barbed ends generated by other means, such as gelsolin- or cofilin-mediated severing, thereby exponentially doubling the number of actin filament barbed ends at each branching.

The Arp2/3 complex contributes to the burst of actin assembly that follows platelet activation, associates with the actin cytoskeleton, and redistributes rapidly and uniformly to the lamellar edge of spread platelets (Falet et al. 2002a, b). It is unlikely that the Arp2/3 complex associates to the tip of platelet filopodia, as has been described in one study (Li et al. 2002), because actin filaments in filopodia are bundled, not branched. The Arp2/3 complex contribution to actin filament nucleation in platelets importantly requires free barbed ends generated by gelsolin-mediated severing, capping, and uncapping (Fig. 2) (Falet et al. 2002b). The Arp2/3 complex clusters in marginal actin filament clumps in *Gsn*-null platelets and fibroblasts, consistent with few uncapped ATP-bound actin-containing barbed ends generated in the absence of gelsolin, placing gelsolin function upstream of Arp2/3 complex nucleation in these cells (Falet et al. 2002b).

The Arp2/3 complex is also recruited to sites of clathrin-mediated endocytosis in megakaryocytes and is responsible for the accumulation of clathrin-coated vesicles and F-actin clusters in megakaryocytes lacking the large GTPase dynamin 2 (DNM2) (unpublished observations). DNM2

normally mediates the fission of endocytic vesicles from the plasma membrane (Ferguson and De Camilli 2012). Mutations in DNM2 have been associated with mild thrombocytopenia and neutropenia in patients with Charcot–Marie–Tooth disease (Züchner et al. 2005). Mice specifically lacking DNM2 in the megakaryocyte lineage develop severe macrothrombocytopenia, megakaryocyte hyperplasia, myelofibrosis, extramedullary hematopoiesis, and severe and rapid splenomegaly due to impaired endocytosis in megakaryocytes and platelets (Bender et al. 2015a).

WASp Family and Actin Nucleation-Promoting Factors

Activation of the Arp2/3 complex requires actin nucleation-promoting factors (NPFs), such as WASp family proteins, which integrate signals leading to actin assembly (Pollard and Borisy 2003). WASp (human *WAS*, mouse *Was*) is the protein mutated in Wiskott–Aldrich syndrome (WAS), a rare X-linked recessive disorder characterized by severe immunodeficiency, eczema, and thrombocytopenia. The disease affects most non-erythroid hematopoietic lineages, including lymphocytes, monocytes, neutrophils, and platelets, which are among the most severely affected cells. X-linked thrombocytopenia (XLT) is a milder form of WAS, in which platelets are primarily affected. Platelets from WAS and XLT patients are abnormally small, and circulating platelet counts can be 10 % of normal or lower. The defect is likely due to increased platelet clearance, as WASp deficiency in megakaryocytes induces premature proplatelet formation and platelet production in the bone marrow compartment (Haddad et al. 1999; Sabri et al. 2006).

In platelets, WASp is present in a constitutive 1:1 complex with WASp-interacting protein (WIP; human *WIPF1*, mouse *Wipf1*) (Falet et al. 2009), and *WAS* mutation hotspots map to the WIP-binding region in WASp and disrupt the WIP–WASp complex interaction (Luthi et al. 2003). A homozygous mutation in *WIPF1* has been reported in one patient to cause recurrent infections, eczema, and severe thrombocytopenia, all features of WAS (Lanzi et al. 2012). Cells isolated from the patient have undetectable WASp, but normal *WAS* sequence and mRNA levels. *Wipf1*-null mouse platelets lack WASp, and WIP expression is reduced in *Was*-null mouse platelets, indicating that both proteins are required for the stability of each subunit of the complex (Falet et al. 2009). *Was*-null and *Wipf1*-null mice have mild thrombocytopenia, and their platelets have normal size (Snapper et al. 1998; Zhang et al. 1999; Falet et al. 2009).

The role of the WIP–WASp complex in platelet shape change and actin assembly is unclear, as platelets lacking either WASp or WIP do not show any defect in actin assembly and shape change (Gross et al. 1999; Rengan et al. 2000; Falet et al. 2009), and the Arp2/3 complex redistributes normally to the actin cytoskeleton and to the edge of lamellipodia in the absence of WASp (Falet et al. 2002a). These observations suggest that platelets differ from the other hematopoietic cells affected by WASp deficiency, raising two fundamental questions. First, what is the role of WASp in platelets? Second, what is the identity of the NPFs activating the Arp2/3 complex in platelets? Recent studies have shown that human and mouse platelets lacking WASp contain abnormally organized and hyper-stable microtubules, suggesting an unexpected role of WASp as a regulator of microtubule organization (Bender et al. 2014). WASp also controls the delivery of platelet transforming growth factor-β_1 (TGF-β_1) (Kim et al. 2013) and regulates the formation of specific platelet actin structures, called actin nodules, which likely play a role in the early stages of platelet spreading (Calaminus et al. 2008; Poulter et al. 2015).

Platelets express other WASp family proteins and NPFs unrelated to WASp, which could theoretically activate the Arp2/3 complex. These include the ubiquitous WASp paralog neural WASp (N-WASP), WASp family verprolin homologs 1 (WAVE1) and 2 (WAVE2), cortactin, and hematopoietic lineage cell-specific protein 1 (HS1). An SNP in *WASL* encoding N-WASP has been associated with altered platelet counts (Soranzo et al. 2009; Gieger et al. 2011). However, the role of N-WASP in platelet production and function, particularly in Arp2/3 complex activation, is unclear. N-WASP can be detected in platelet lysates by immunoblot analysis (Shcherbina et al. 2001; Falet et al. 2002a), but its expression appears to be low because platelet lysates fail to initiate the Arp2/3 complex-dependent motility of bacteria expressing *Shigella flexneri* IcsA, which normally recruits and requires N-WASP in host cells (Egile et al. 1999).

WAVE1 (human *WASF1*, mouse *Wasf1*) and WAVE2 (human *WASF2*, mouse *Wasf2*) localize at the edge of lamellipodia and at the tips of filopodia in spread platelets (Kashiwagi et al. 2005). WAVE2 deficiency is lethal by embryonic day 12.5 in mice. *Wasf2*-null mouse megakaryocytes differentiated from embryonic stem cells are severely impaired in terminal differentiation, spreading onto fibrinogen and platelet production (Eto et al. 2007). By contrast, WAVE1 does not appear to be required for platelet production in vivo, as *Wasf1*-null mice have normal platelet counts (Eto et al. 2007), but is critical for platelet spreading and cytoskeletal reorganization downstream of the collagen receptor GPVI (Calaminus et al. 2007). Interestingly, *Wasf1*-null mouse platelets respond normally to stimulation by G-protein coupled receptors, indicating that platelet WAVE1 plays a signaling role in the GPVI signaling cascade, rather than a cytoskeletal role. Early studies have shown that cortactin redistributes to the cortex of spread platelets (Ozawa et al. 1995), similar to the Arp2/3 complex. However, cortactin and its hematopoietic specific paralog HS1

appear to be dispensable for platelet production, spreading, and lamellipodia formation in mice (Thomas et al. 2007; Schnoor et al. 2011).

Based on these observations, N-WASP and WAVE2 appear to be the best candidates for further investigation in their contribution to Arp2/3 complex activation in platelet production and function. N-WASP is believed to activate Arp2/3 complex-mediated actin nucleation activity to induce filopodia formation downstream of the small GTPase Cdc42, whereas WAVE2 orchestrates lamellae spreading downstream of Rac1 (Aslan and McCarty 2013). Rac1 deletion in platelets leads to impaired lamellipodia spreading, as well as to altered responses to GPVI stimulation due to defective phospholipase C-γ2 (PLC-γ2) activation (McCarty et al. 2005; Pleines et al. 2009, 2013). By contrast, platelets lacking Cdc42 display normal filopodia formation, but enhanced secretion and increased aggregation in response to agonist stimulation (Pleines et al. 2010). Platelet-specific Cdc42 deletion also leads to thrombocytopenia, due to impaired DMS formation and platelet production by megakaryocytes (Pleines et al. 2013).

Actin Nucleation by Formins

Formins are another group of proteins regulating actin nucleation (Goode and Eck 2007). Formins are involved in various cellular functions such as cell polarity, motility, and cytokinesis. At cellular levels, formins are required for assembly of stress fibers, cytoplasmic actin networks used for vesicle transport, cytokinetic actin rings, and phagocytic cups. Formins are multidomain proteins that interact with diverse signaling molecules and cytoskeletal proteins. Formins associate with the fast-growing barbed end of actin filaments via their signature formin homology 2 (FH2) domain. Formins initiate filament assembly and remain persistently attached to the barbed end without dissociating, enabling rapid insertion of actin subunits while protecting the end from capping proteins. Formins generate single actin filaments that are typically oriented orthogonally to membranes. Formins have also been shown to directly regulate microtubule dynamics (Chesarone et al. 2010).

Fifteen formin isoforms are present in humans, classified in seven subgroups. The most predominantly expressed diaphanous-related formins are autoinhibited through intramolecular interactions and appear to be activated by the small GTPase RhoA and additional factors. Other classes of formins lack the autoinhibitory and/or RhoA-binding domains and thus are likely to be controlled by alternative mechanisms. Three formins have been characterized biochemically in platelets: diaphanous homolog 1 (DIAPH1; human *DIAPH1*, mouse *Diaph1*), disheveled-associated activator of morphogenesis 1 (DAAM1; human *DAAM1*, mouse *Daam1*), and FH1/FH2 domain-containing protein 1 (FHOD1; human *FHOD1*, mouse *Fhod1*) (Higashi et al. 2008; Thomas et al. 2011). Based on profiling studies, platelets also appear to abundantly express inverted formin 2 (INF2; human *INF2*, mouse *Inf2*). However, its role in platelet formation and function has not yet been investigated.

DIAPH1 and DAAM1 purified from platelet extracts assemble actin in the presence of RhoA, suggesting that they contribute to actin dynamics in activated platelets downstream of RhoA (Higashi et al. 2008). A gain-of-function mutation in DIAPH1 has been associated with dominant macrothrombocytopenia (Stritt et al. 2016). DIAPH1 knockdown increases proplatelet formation by $CD34^+$ cell-derived human megakaryocytes by decreasing their F-actin content, but increasing tubulin polymerization and stability (Pan et al. 2014). Inversely, DIAPH1 overexpression in megakaryocytes increases stress fiber formation. Interestingly, analysis of *Diaph1*-null mice reveals no alteration in platelet counts or platelet hemostatic function in response to stimulation, including spreading and clot retraction (Thomas et al. 2011), although mice lacking platelet RhoA develop macrothrombocytopenia (Pleines et al. 2012). *Diaph1*-null mice develop age-dependent myeloproliferative/myelodysplastic phenotypes, including splenomegaly, fibrotic and hypercellular bone marrow, and extramedullary hematopoiesis in both spleen and liver, suggesting that DIAPH1 acts as a tumor suppressor (Peng et al. 2007). FHOD1 undergoes rapid phosphorylation downstream of ROCK in activated platelets and may therefore be involved in the formation of stress fibers (Thomas et al. 2011). Further studies are required to establish the physiological role of formins in platelet actin dynamics.

Conclusion

The platelet cytoskeleton has two main functions: (1) maintain the discoid shape of the resting platelet and (2) mediate a rapid shape change in response to external stimuli at sites of vascular injury. At rest, specific cytoskeletal structures, such as the spectrin-based membrane cytoskeleton and cytoplasmic actin filaments cross-linked by FlnA or bundled by α-actinins, maintain the unique architecture of the platelet actin cytoskeleton and connect it to the plasma membrane. Actin monomer-sequestering proteins, such as thymosin β_4, and actin filament capping proteins, such as CapZ, prevent the assembly of actin monomers onto the barbed ends of the actin filaments composing the resting cytoskeleton. Platelet activation leads to a rise in intracellular calcium and synthesis of membrane PIP2 to stimulate the activities of actin

regulatory proteins, such as gelsolin and the Arp2/3 complex. The interactions of these cytoplasmic signals and molecules initiate and amplify actin filament growth necessary for platelet shape change.

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Take-Home Messages

The platelet cytoskeleton: (1) maintains the discoid shape of the resting platelet and (2) mediates a rapid shape change in response to external stimuli at sites of vascular injury.

In resting platelets: (1) the spectrin-based membrane cytoskeleton and cytoplasmic actin filaments cross-linked by FlnA or bundled by α-actinins maintain the unique architecture of the platelet actin cytoskeleton and connect it to the plasma membrane and (2) actin monomer-sequestering proteins such as thymosin β_4 and actin filament capping proteins such as CapZ prevent the assembly of actin monomers onto the barbed ends of the actin filaments composing the resting cytoskeleton.

In activated platelets: a rise in intracellular calcium and synthesis of membrane PIP2 stimulate the activities of actin regulatory proteins, such as gelsolin and the Arp2/3 complex, to initiate and amplify actin filament growth necessary for platelet shape change.

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Platelet Proteomics and its Applications to Study Platelet-Related Disorders

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Abstract

Proteomics is a rapidly evolving research approach in parallel with genomics, utilizing advanced technologies in protein separation, identification, quantification, and bioinformatics. We have reviewed the different proteomic techniques that have been used successfully to analyze platelets in humans over time. Using more advanced technologies, an increasing number of platelet proteins have been identified and this by investigating the resting platelet proteome and that of activated platelets using diverse agonists. It is also possible to analyze platelet subproteomes including that of granules, microparticles, and membrane proteins. Furthermore, experiments can be designed that specifically study changes in the phosphorylation, glycosylation, or palmitoylation profiles of platelet proteins. These studies have generated extensive protein databases of >5000 proteins for platelets under normal physiological conditions that are also useful to study disease. Examples of proteomic studies that were designed to study platelet-related bleeding disorders and cardiovascular diseases, but also other complex disorders where platelets can be used as a model cell, are discussed.

Introduction to Platelet Proteomics

Proteomics is the large-scale study of proteins encoded by a genome under specific conditions and at a given time (Wilkins et al. 1996) and has also been used to study platelets and platelet-related diseases. Because of recent advances, especially in mass spectrometry (MS), proteomics now enables the measurement of multiple properties for thousands of proteins simultaneously, such as their abundance, modifications, subcellular localization, and protein–protein interactions (Larance and Lamond 2015). Platelets are estimated to contain about 5000 different proteins, spanning a wide abundance range and with different post-translational modifications (Burkhart et al. 2012). The platelet proteome is highly dynamic as some proteins are secreted upon platelet activation and the proteome itself changes with age (Cini et al. 2015) or disease state (Macaulay et al. 2005). In addition, the capability of platelets to absorb plasma proteins further increases the proteome variability and complexity. Platelets are anuclear, having no DNA and only limited amounts of mRNA, which makes proteomic research an attractive alternative for gene expression studies. Platelets are readily available in living organisms and in relatively high amounts; therefore proteomes can also be applied to analyze platelets from children starting from small volumes of blood. As for other cell types and organisms, knowledge of the proteome is expected to be more useful than information on the transcriptome as proteins are believed to be the main effectors of gene functions. It has been estimated that the 20,000 human genes translate into one million different proteoforms due to alternatively splicing and posttranslational modifications (Brett et al. 2002). Furthermore, several

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studies have revealed a poor correlation between mRNA and protein profiles in lower organisms (Gygi et al. 1999a, b), and this has also been described for platelets (Burkhart et al. 2012; Londin et al. 2014). This discrepancy might be explained by a rapid decay of mRNAs in response to various stimuli and posttranslational protein ubiquitination leading to protein degradation. Moreover, a disease state may be determined by translocation of proteins to different cellular compartments rather than by changes in transcript levels. In addition, several technical issues can explain the imperfect correlation between transcriptomic and proteomic data. For example, the current transcriptomic techniques provide only limited information on alternative splicing, whereas proteomics can detect separate protein isoforms encoded by these variants. Given all these considerations, proteomics represents an efficient tool to gain deeper insights into the molecular mechanisms regulating unknown platelet functions and platelet-related diseases. We, and others, published extensive reviews on the application of proteomics in the field of platelet research, mainly focused on the commonly used methodologies and the key achievements in the elucidation of platelet-related diseases (Di Michele et al. 2012a, b; Zufferey et al. 2012; Rotilio et al. 2012). Here, we summarize recent updates for the main contemporary proteomic strategies for platelet research in humans and the most important studies that applied proteomics to unravel the molecular mechanisms underlying platelets in physiological ("Proteomic Methods Used for Platelet Research" and "Proteomic Strategies to Decipher Platelet Biology") and pathological conditions ("Platelet Proteomics to Gain Insights in Human Diseases").

Proteomic Methods Used for Platelet Research

Gel-Based Proteomics

Gel-based methods, including conventional 2DE (bidimensional electrophoresis) and DIGE (differential in-gel electrophoresis), are commonly used for platelet proteomics. In 2DE, proteins are first separated based on their isoelectric point along a pH gradient by isoelectric focusing and then based on their apparent molecular mass in a polyacrylamide gel by SDS-PAGE (Rogowska-Wrzesinska et al. 2013). Proteins are visualized by in-gel staining, matched in different 2DE gels, quantified, and proteins of interest are excised for further identification by MS. 2DE was first used to study the platelet proteome in the 1970s, identifying some most abundant proteins and membrane glycoproteins (Clemetson et al. 1979) and later for the identification of proteins with changed levels upon platelet storage (Snyder et al. 1987). This method also contributed to generate the proteome reference maps of quiescent platelets (Gravel et al. 1995; Marcus et al. 2000; O'Neill et al. 2002; Garcia et al. 2004a, b), activated platelets (Claeys et al. 2005; Shai et al. 2012), and platelet subproteomes including microparticles (Shai et al. 2012; Garcia et al. 2005), cytosol, and microsomes (Claeys et al. 2005). 2DE holds intrinsic limitations such as the difficulty to analyze hydrophobic, extremely charged, and very small or large proteins, as well as having a limited reproducibility among gels and the presence of multiple proteins in a single spot. Combined with restricted sample loading and the scarce sensitivity of protein stains, all of this hampers the detection of low-abundant proteins. Some of these disadvantages were overcome using DIGE, which allows improved reproducibility, higher quantification accuracy, and reduced time/costs. With DIGE, two samples and one internal standard are loaded together on the same gel after labeling with different fluorescent dyes (Cydye-2, Cydye-3, and Cydye-5), each with specific excitation/emission wavelengths (Unlu et al. 1997). The internal standard is a pool of equal amounts of all the samples used to facilitate accurate spot matching across gels, minimizing inter-gel variability, and allowing to calculate the abundance of each protein spot as a ratio to its corresponding spot in the internal standard, leading to the measurement of subtle changes in protein abundance with high statistical confidence. DIGE has been used to study platelet proteomes under basal or activated conditions (Cini et al. 2015; Baumgartner et al. 2013; Winkler et al. 2008; Veitinger et al. 2012).

Finally, 1D SDS-PAGE can be used in combination with MS in the so-called GeLC-MS approach (Shevchenko et al. 2006). The underlying workflow involves SDS-PAGE, in-gel protein staining, slicing of each lane into discrete gel bands, in-gel protein digestion, peptide purification, and LC-MS/MS analysis. GeLC-MS was successfully used to study human platelet proteomes in resting and stimulated conditions (Piersma et al. 2009; Qureshi et al. 2009; van den Bosch et al. 2014; Ambily et al. 2014), during storage (Dzieciatkowska et al. 2015a; Schubert et al. 2012a; b), or to analyze specific subproteomes including the immunoproteasome (Klockenbusch et al. 2014) and palmitoylome (Dowal et al. 2011).

Gel-Free Proteomics

Gel-based methods are progressively replaced by so-called bottom-up MS-based proteomic methods that analyze peptide mixtures derived from isolated proteomes. These techniques require lower amounts of material, which is particularly important when studying rare platelet-related diseases, platelets from young children, or subproteomes. The first step in gel-free analysis typically consists of digesting the proteome with a specific protease. This is

followed by a chromatographic separation of the obtained peptides coupled to MS for analysis, subsequent data processing, and peptide identification.

The pioneering method is multidimensional protein identification technology (MudPIT) which combines orthogonal peptide separations prior to MS/MS analysis, thereby maximizing sensitivity and resolution (Washburn et al. 2001). The main advantage of MudPIT over gel-based methods is that it largely avoids protein solubilization problems as proteins are cut into peptides that are easier to solubilize. MudPIT was used to analyze the basal platelet proteomes (Finamore et al. 2010), the thrombin-induced secretome (Coppinger et al. 2004), the serine hydrolase subproteome (Holly et al. 2013), and the effects of dysregulated miRNA on thrombocythemic platelets (Xu et al. 2012).

The exponentially modified protein abundance index (emPAI) approach is widely used and assumes that abundant peptides are more often detected, but also considers that bigger proteins and proteins with many peptides in the m/z-range for efficient MS analysis will generate more observed peptides (Ishihama et al. 2005). The emPAI method was successfully employed when studying protein networks associated with platelet glycoprotein VI stimulation (Wright et al. 2011).

Combined fractional diagonal chromatography (COFRADIC) is a gel- and label-free proteomic method (Gevaert et al. 2002). Given its versatility, several COFRADIC protocols were developed, each allowing for the enrichment of a specific set of peptides (Gevaert et al. 2003; Stes et al. 2014; Staes et al. 2011). COFRADIC relies on three steps: (1) a first RP-HPLC fractionation of the digested proteome, (2) a chemical or enzymatic reaction of single or combined peptide fractions that modifies the chemical structure of selected peptides that will then obtain altered chromatographic properties, and (3) a series of RP-HPLC fractionations identical to the first one during which the altered peptides are isolated for LC-MS/MS analysis. COFRADIC was applied for studying the human platelet proteome together with GeLC-MS/MS and MudPIT, revealing that the use of complementary techniques allows for more comprehensive proteome mapping (Martens et al. 2005; Lewandrowski et al. 2009).

Labeling methods rely on the incorporation of stable isotopes, i.e., ^{2}H, ^{13}C, ^{15}N, and ^{18}O by metabolic (SILAC), chemical (ICAT, iTRAQ, or TMT), or enzymatic reactions. In stable isotope labeling by amino acids in culture (SILAC), cells are grown separately in media supplemented with heavy or light labeled essential amino acids, such as lysine and arginine (Ong et al. 2002). Although SILAC is typically only applied to cells that can be expanded in vitro, it was also successfully used for studying platelet proteomes (Kruger et al. 2008; Zeiler et al. 2014). In isobaric tags for relative and absolute quantitation (iTRAQ), quantification occurs using MS/MS spectra rather than MS spectra (Gygi et al. 1999a, b). iTRAQ makes use of an amine-reactive reagent available in different isotopic variants to label peptides at their primary amines. iTRAQ was used to analyze the proteome of human platelets focusing on inter- and intra-biological variations (Burkhart et al. 2012; Vaudel et al. 2012) and platelets stimulated with ADP, thrombin, collagen, TRAP, and/or iloprost (Beck et al. 2014; Cimmino et al. 2015). Furthermore, iTRAQ-based gel-free proteomics, combined with DIGE- and ICAT-based proteome analysis, was applied to detect changes in platelet protein levels during storage of blood products (Thon et al. 2008). Similar to iTRAQ, tandem mass tags (TMTs) employ amine-reactive chemicals to label peptides (Thompson et al. 2003), and up to ten different isobaric tags are available (Werner et al. 2014). TMT labeling was applied to study protein changes in platelets from subjects sensitive or resistant to aspirin (Floyd et al. 2014). Enzymatic labeling is based on peptide labeling with oxygen-18 isotopes (Yao et al. 2001). Stable enzymatic labeling of tryptic peptides in combination with COFRADIC was applied to analyze platelets (Staes et al. 2004).

Proteomic Strategies to Decipher Platelet Biology

General Considerations for Experimental Design of Platelet Proteomic Studies

Most proteomic studies are feasible with about 5 mL of blood allowing the isolation of 10^8 platelets, corresponding to 1 mg of proteins (Burkhart et al. 2012). For comparative proteomic studies, different groups should contain age- and sex-matched donors. Indeed, a DIGE study reported a quantitative variation of 18 % in platelet proteomes of a group of 56–100 years old volunteers, probably due to age differences (Winkler et al. 2008). Also another study detected important differences in the platelet proteome of children and adults using DIGE coupled to LC-MS/MS (Cini et al. 2015). Moreover, higher levels of signaling cascade proteins have been found by 2DE and MS in male compared to female donors (Eidelman et al. 2010). Significant gender differences in platelet proteins have also been detected in a recent study using QconCAT-based MRM, not only in fresh blood from donors but also in apheresis platelet concentrates following varying storage times (Dzieciatkowska et al. 2015b). Finally, inter- and intra-subject variation among the human platelet proteome was measured in four healthy donors and three different blood samples from one donor and revealed that the proteome showed about 15 % of quantitative variation among donors (Burkhart et al. 2012). These studies

highlight the usefulness of including biological replicates in the experimental setup to counterbalance for the intrinsic variability of samples. In addition, also some other factors might be considered when choosing platelet donors, such as medication use (such as aspirin), lifestyle, and dietary habits. Furthermore, cigarette smoking was also found to differentially regulate the levels of several platelet proteins (Della Corte et al. 2012).

Obviously, protocols for sample preparation are also a non-negligible source of variability. For example, the analytical variation of a single sample of platelet proteins within and between four laboratories each using its own 2DE protocol was measured, and the coefficient of variation for each of the matched spots after automatic and subsequent manual matching ranged between 5 and 60 % (de Roos et al. 2008). A 2DE study also revealed changes in the platelet proteome in terms of composition rather than yield depending on the precipitation method used to purify proteins, namely, ethanol versus trichloroacetic acid (Zellner et al. 2005). The type of anticoagulant (acid citrate dextrose, heparin, EDTA, etc.) used for blood collection is of fundamental importance, as these substances can induce changes by interacting with specific platelet proteins (Capila and Linhardt 2002). Particular attention must be given to obtaining highly purified platelet fractions, due to possible contamination mainly from plasma and other blood cells. The origin of plasma proteins found in platelet proteomes, however, is still a matter of debate as it is not clear if they derive from the sample preparation method or are actually located within platelets given their surface-connected open canalicular system and its continuous exchange with plasma components. A shotgun proteome analysis of the cytoplasmic, microparticle, and secreted fractions of TRAP-activated platelets supports the platelet origin of some plasma proteins identified in platelet proteomes, thus excluding methodological artifacts (Veitinger et al. 2012). Concerning contamination with other blood cells, a protocol for isolating highly pure platelets preparations is recommended, leading to platelet preparations with less than 1 leukocyte per 10^6 platelets and less than 1 erythrocyte per 10^4 platelets, beyond <1.5 % by volume of plasma per platelet (Burkhart et al. 2012; Gambaryan et al. 2010). In this context, particularly useful are freely available protein databases of purified blood cells (including T cells, monocytes, neutrophils, erythrocytes, and platelets) obtained by both 2DE and shotgun proteomics, which may be used as references (Haudek et al. 2009).

Proteomics of Resting Platelets

Since the first attempts to characterize the human platelet proteome in the late 1970s by 2DE (Clemetson et al. 1979), a huge number of such proteome studies have followed that have identified a growing number of proteins. A combination of 2DE, immunoblotting, and N-terminal sequencing identified about 25 platelet proteins (Gravel et al. 1995). The cytosolic fraction of platelets was analyzed in a successive study using 2DE combined with MS, leading to the identification of more than 200 proteins (Marcus et al. 2000). Two 2DE studies in combination with improved first dimension separation identified more than 500 and 300 different proteins, respectively (O'Neill et al. 2002; Garcia et al. 2004a, b). COFRADIC gave a further impulse to the development of a detailed platelet proteome map of about 650 platelet proteins, including several proteins not previously detected, and hydrophobic membrane proteins (Gevaert et al. 2003). Another study based on 2DE and shotgun proteomics contributed to the compilation of the platelet proteome in basal conditions by identifying about 100 unique proteins (Finamore et al. 2010). Shotgun proteomics, based on peptide separation by OFFGEL fractionation and RP chromatography coupled to LC-MS/MS detection, was also applied to platelet proteomics and led to the identification of more than 1300 proteins (Krishnan et al. 2011). Finally, the most comprehensive proteome database of human platelets consists of more than 4000 unique (phospho)proteins and has been obtained by iTRAQ and TiO_2 enrichment for the phosphorylated fraction (Burkhart et al. 2012).

Proteomics of Activated Platelets

Upon activation by endothelial cell damage or inflammatory events, platelets release a high number of proteins into the circulation, the so-called platelet secretome or releasate, which is involved in the regulation of primary hemostasis, but also in coagulation, inflammation, angiogenesis, wound healing, and some other processes (Golebiewska and Poole 2015). Numerous proteomic studies have attempted to characterize the platelet secretome using different methods. Moreover, different agonists have been used to activate platelets and induce the protein releasate, such as ADP, thrombin, TRAP, collagen, and arachidonic acid. These agonists have been used alone or in combination, influencing the composition of platelet secretome (Rogowska-Wrzesinska et al. 2013). A concentration step on reverse-phase chromatographic beads of diluted peptide mixtures following in-gel digestion of 2DE-separated proteins and MALDI-TOF-MS analysis was applied to the proteome of platelets activated by thrombin with the identification of several proteins that translocate to the cytoskeleton fraction (Gevaert et al. 2000). In another study, a combination of 2DE and MudPIT resulted in a comprehensive characterization of the secretome of thrombin-activated platelets, leading to more than 300 identified proteins, many of which were

described for the first time in platelets, such as secretogranin III, cyclophilin A, and calumenin (Coppinger et al. 2004). Another MudPIT-based analysis led to the identification of 82 proteins differentially expressed in the platelet secretome upon stimulation with thrombin (McRedmond et al. 2004). In activated platelets, these proteins include chemokines, signaling molecules, and histones, as well as coagulation factors and receptors involved in thrombosis. Another study aimed at characterizing the proteome of thrombin-stimulated platelets using DIGE coupled to MALDI-TOF-MS (Della Corte et al. 2008). By comparing the secretome of unstimulated and stimulated platelets, several differentially secreted proteins were identified, including laminin A, a nuclear protein described for the first time as released by platelets. A recent study using DIGE and LC-MS/MS focused on the difference in the platelet secretome following stimulation with thrombin or collagen (Vélez et al. 2015). Thirty-eight proteins differentially present between these conditions were identified, and this relatively high number of protein differences confirms the influence of the type of agonist used for platelet stimulation on the composition of the secretome. The analysis of the platelet proteome in resting or TRAP-activated conditions by 2DE led to the identification of 31 regulated proteins (Garcia et al. 2004a, b). Among these, eight were not known to be present in platelets, including the adapter downstream of tyrosine kinase 2 (DOK-2). The number of proteins identified in the secretome of TRAP-stimulated platelets dramatically increased when using a GeLC-MS/MS approach, based on 1D SDS-PAGE and LTQ-FT MS with the identification of 716 proteins (Piersma et al. 2009). About 40 % of these proteins were identified for the first time in platelet releasates.

Platelets were also stimulated with less common agonists such as GPIV-activating collagen receptor peptide (CRP) (Wright et al. 2011). In this study, different platelet subcellular compartments were isolated using ultracentrifugation to reduce the overall complexity. The majority of differentially expressed proteins after CRP stimulation were low abundant and involved in signaling. In addition, more than half of the 663 identified proteins were not previously known to be present in platelets. An accurate quantitative proteomic strategy based on stable isotope dimethylation, SCX, and MS analysis was used to compare the platelet secretome following PAR1 or PAR4 stimulation or in resting conditions (van Holten et al. 2014). The differential release of pro- and anti-angiogenic growth factors by PAR1 and PAR4 might be important for the regulation of angiogenesis, though no large differences in protein abundance could be detected that confirmed such a hypothesis.

Some proteomic studies also investigated the effect of different agonists simultaneously on the platelet proteome. 2DE combined with LC-MS/MS was used to identify 144 platelet proteins with changing levels following activation with arachidonic acid, collagen, and thrombin (Majek et al. 2010). A more recent study analyzed the platelet secretome following thrombin and collagen treatment using a reversed releasate approach (Wijten et al. 2013). Contrary to classical studies on platelet secretomes, which may also detect proteins derived from cell lysis, in this case the protein levels in the platelets after stimulation were accurately quantified following stable isotope labeling, assuming that the released proteins are less abundant in the platelet proteome. Importantly, of the about 4500 platelet proteins quantified, only 124 were found to be secreted, spanning a concentration range of $\geq$5 orders. These proteins included several novel low-abundant proteins and well-known proteins such as thrombospondin and von Willebrand factor.

Analysis of Platelet Subproteomes

Platelet Granules

To reduce the complexity of analyzing total proteomes, the isolation of organelles prior to analysis is often used. Organelle isolation can be achieved via density gradient centrifugation, immunopurification, or free-flow electrophoresis (Zufferey et al. 2012). However, assessment of possible contamination from other organelles is recommended prior to analysis using organelle markers via immunoblotting, electron microscopy, or enzymatic assays. Platelets contain three types of secretory granules, alpha granules, dense granules, and lysosomes, each characterized by a different number per platelet, content, morphology, and response to stimuli (Rendu and Brohard-Bohn 2001). The proteome of alpha granules isolated by sucrose gradient ultracentrifugation was extensively characterized by GeLC-MS/MS, leading to the identification of 219 proteins (Maynard et al. 2007). Not surprisingly, the majority of these were already described in platelet secretomes, while 44 proteins were novel. The proteome of dense granules was analyzed using two proteomic methods: 2DE coupled to MALDI-TOF-MS and LC-MS/MS analysis (Hernandez-Ruiz et al. 2007). Overall, 40 proteins were identified, and most of them, such as actin-associated proteins, glycolytic enzymes, and regulatory proteins, were not previously known to reside in these organelles. More recently, a proteomic analysis carried out by subcellular fractionation on a sucrose gradient and MS analysis led to the most comprehensive characterization of the platelet granule proteome, identifying over 800 proteins (Zufferey et al. 2014).

Platelet Microparticles

In the most comprehensive analysis of platelet microparticles so far, almost 600 proteins were identified

(Garcia et al. 2005). About 65 % of these proteins have been described for the first time, suggesting that these organelles have a unique protein composition. The proteome of platelet microparticles was also analyzed in a subsequent study using 2DE and LC-MS/MS, in which the microparticles were separated by gel filtration in four classes based on their size (from approximately 100 nm to greater than 500 nm), revealing major differences in protein composition (Dean et al. 2009). In particular, mitochondrial proteins were mostly present in the largest microparticles, whereas alpha granule proteins were more found in the smallest microparticle fractions. The platelet microparticles proteomes were different depending on the stimulus used to activate platelets as found by another proteomic study using 2DE (Shai et al. 2012). Twenty-six proteins were differentially expressed between shear- and thrombin-activated platelets, mainly involved in signaling pathways. The proteome of microparticles from ADP-stimulated platelets was also extensively characterized by shotgun proteomics (Capriotti et al. 2013). This approach led to the identification of more than 600 proteins, 40 % of which were described in platelet microparticles for the first time.

Platelet Plasma Membrane

Despite a major role for membrane receptors and associated proteins for platelet function, they are relatively poorly represented in proteomics studies. This is mainly due to the characteristics of these proteins, such as their low abundance, hydrophobicity, and the reduced accessibility of trypsin to transmembrane domains, which hamper both gel- and MS-based proteomics. Therefore, specific methods are required to improve plasma membrane protein identification. The first study focusing on the platelet plasma membrane proteome used pre-fractionation over a sorbitol gradient to remove high abundant cytoskeletal proteins and contaminants prior to plasma membrane isolation and GeLC-MS/MS analysis (Moebius et al. 2005). In addition, 1D SDS-PAGE separation was performed both by conventional SDS-PAGE and benzyldimethyl-n-hexadecylammonium chloride/SDS separation to improve protein resolution on gel. Almost 300 proteins could be identified, the majority of which were plasma membrane proteins. In a subsequent study, three different techniques were used for the enrichment of platelet plasma membrane proteins before LC-MS/MS analysis, namely, lectin affinity chromatography, biotin/NeutrAvidin affinity chromatography, and free-flow electrophoresis (Senis et al. 2007). A total of 136 membrane proteins were identified, many of which previously not known as platelet membrane proteins. An interesting comparative analysis of the membrane proteomes from platelets in control and thrombin-activated conditions was carried out by NeutrAvidin affinity chromatography, prior to protein separation by liquid-phase IEF and SDS-PAGE and analysis by FT-ICR MS to identify 88 differentially expressed proteins (Tucker et al. 2009). Another study identified 182 membrane proteins using a different proteomic approach (Qureshi et al. 2009). Membrane proteins were first precipitated via ultracentrifugation, resuspended in a glucopyranoside/guanidium buffer and subsequently analyzed by LC-MS/MS. The most extensive proteomic study on platelet plasma membrane proteins led to the identification of 1282 proteins, which were also relatively quantified via an emPAI-based method (Lewandrowski et al. 2009). The power of this study resides in the use of three different proteomic approaches, GeLC-MS/MS, MudPIT, and N-terminal, methionine, or cysteine COFRADIC, all in combination with aqueous two-phase partitioning.

Proteomics to Analyze Platelet Posttranslational Modifications

Platelet Phosphoproteome

Phosphoproteomics is particularly useful for studying cellular signaling events. 2DE and radioactive labeling were used to identify phosphoproteins upon thrombin activation (Immler et al. 1998). Several protein spots showing a significant increase or decrease in phosphorylation could be detected, in particular different myosin isoforms. Another study was also conducted on thrombin-stimulated platelets, focusing on tyrosine phosphorylation, which plays a central role in platelet activation (Maguire et al. 2002). Proteins containing phosphorylated tyrosine were immunoprecipitated using a monoclonal antibody, separated by 2DE, and the resulting protein spots differentially found between platelets in control and activated conditions were identified by immunoblotting and MALDI-TOF-MS. Further development of modern and more resolving techniques for phosphoprotein/peptide enrichment and MS analysis significantly contributed to shed light on signal-dependent activation responses (Zahedi et al. 2006). For example, two different approaches, treatment with ^{32}P followed by 2DE using different pI ranges and autoradiography or immunoprecipitation coupled to GeLC-MS/MS analysis, led to the identification of 55 phosphoproteins and some in vivo phosphorylation sites (Marcus et al. 2003). In another work, using IMAC-based phosphopeptide enrichment and SCX chromatography coupled to LC-MS/MS, 564 phosphorylation sites belonging to almost 280 proteins were identified in resting platelets (Zahedi et al. 2008). An extensive analysis of the proteome and phosphoproteome of platelets in resting conditions, with particular attention to the integrin signaling pathway, was undertaken by IMAC and GeLC-MS/MS (Qureshi et al. 2009). This led to the identification of more than 1500 proteins, including 262 phosphoproteins. Another strategy for phosphoprotein identification is based on the

enrichment of phosphopeptides via titanium dioxide chromatography and LC-MS/MS after a prior enrichment of plasma membrane proteins in an aqueous two phase (Premsler et al. 2011). In yet another study, protein pulldown using SH2 domains and LC-MS/MS analysis were used to investigate the phosphotyrosine state of platelets upon ADP activation (Schweigel et al. 2013). To study the phosphorylation events mediating platelet activation by collagen, a comparative phosphoproteomic study was carried on phosphotyrosine proteins in resting and CRP-activated platelets (Bleijerveld et al. 2013). This approach was based on immunoprecipitation of phosphotyrosine peptides and stable isotope labeling MS and allowed to identify more than 200 phosphotyrosine sites with an altered phosphorylation status. Also prothrombotic oxidized phospholipids in addition to thrombin were used as agonists for platelet stimulation (Zimman et al. 2014). Here, proteins from stimulated platelets were digested by trypsin, and phosphotyrosine peptides were enriched by immunoprecipitation followed by IMAC. The nonprecipitated proteins were fractionated by SCX, and the resulting fractions further enriched for phosphopeptides using TiO_2 beads. Finally, phosphopeptides were identified and quantified by LC-MS/MS. Interestingly, one study focused on the characterization of the signaling pathways involved in platelet inhibition rather than platelet activation (Beck et al. 2014). Human platelets were treated with iloprost, a stable analog of prostacyclin, which is the most important physiological inhibitor of platelet activation acting on the cAMP/PKA signaling cascade. Using iTRAQ and TiO_2 phosphopeptide enrichment, about 300 phosphopeptides modulated by iloprost were detected.

Platelet Glycoproteome

Studies have shown that platelets possess efficient glycosyltransferase machinery, with more than 200 glycosyltransferases and several substrates (Wandall et al. 2012). The first extensive proteomic study on platelet glycoproteome was performed using concanavalin A affinity chromatography and hydrazide chemistry for enriching glycopeptides, after which they were deglycosylated by N-glycosidase F and analyzed by LC-MS/MS (Lewandrowski et al. 2006, 2009). Concanavalin A, one of the most common lectins in glycoproteomic studies, selectively binds N-glycoproteins. This approach identified 41 glycoproteins and 70 different glycosylation sites. In a subsequent study, the same group focused on the glycosylation sites of platelet membrane proteins (Lewandrowski et al. 2007). The membrane fraction was enriched via aqueous two-phase partitioning in a polyethylene glycol/dextran polymer system, the obtained proteins were digested by trypsin, and the glycopeptides were purified by SCX chromatography prior to N-glycosidase F deglycosylation and MS analysis. By applying this method, almost 150 glycosylation sites on 79 different proteins could be identified, 75 % of which were annotated as plasma membrane proteins. The same group also introduced another strategy for glycopeptide enrichment based on electrostatic repulsion hydrophilic interaction chromatography (Lewandrowski et al. 2008). Importantly, this method might allow resolving different protein isoforms, because of the different interactions of glycopeptides with the stationary phase. By identifying 125 glycosylation sites on 66 different proteins, this work largely contributed to the compilation of an extensive glycosylation site database for human platelet proteins.

Platelet Palmitoylome

Protein palmitoylation, the covalent attachment of long chain fatty acids to cysteines, has an important role in platelet biology, being involved in the regulation of platelet activation and thrombi formation (Sim et al. 2007). A first attempt to characterize the platelet palmitoylome was performed by enriching the platelet membrane protein fraction by acyl-biotinyl exchange chemistry and LC-MS/MS analysis and led to the identification of 215 palmitoylated proteins (Dowal et al. 2011). These proteins included already known palmitoylated proteins, but also 103 new putative palmitoylated ones.

Platelet Proteomics to Gain Insights in Human Diseases

Platelet Proteomics to Study Platelet-Related Bleeding Disorders

Rare inherited platelet-related bleeding disorders are a highly heterogeneous group of disorders that can be caused by abnormal platelet numbers, morphology, and function (Freson et al. 2014). Many genes have been identified as a cause for these disorders, though several patients with an expected platelet-related bleeding disorder still do not receive a genetic diagnosis (Westbury et al. 2015). However, in recent years, next-generation sequencing has offered the potential to improve the diagnosis for such patients. However, in parallel to such genetic studies, proteomics has been used with success to gain insights in such platelet-related bleeding disorders. In 2007, changes in α-granule proteins were quantified for a patient with gray platelet syndrome (GPS) compared to a healthy control (Maynard et al. 2007). GPS is an inherited bleeding disorder caused by a reduced number of α-granules and macrothrombocytopenia. A linear sucrose gradient method was used to isolate α-granules, and proteins were separated by 1D SDS-PAGE and identified by LTQ-FT MS. About 586 proteins were identified, and it was found that soluble, biosynthetic cargo proteins were severely

reduced or undetected in GPS platelets, whereas the packaging of soluble, endocytic cargo proteins was only moderately affected, supporting the defect in α-granule incorporation of proteins synthesized in megakaryocytes. Another bleeding disorder that affects α-granules is Quebec platelet disorder (QPD), characterized by high levels of urinary plasminogen activator (uPA) within platelets. The platelet proteome of four members of the same QPD family was compared to that of two healthy donors using 1D SDS-PAGE gels and LC-MS/MS analysis (Maurer-Spurej et al. 2008). Three α-granule proteins, fibrinogen, multimerin, and thrombospondin-1, were downregulated in patients' platelets, and it was speculated that this was due to degradation by platelet-derived uPA. Combined DIGE and MALDI-ToF/ToF were used to compare the platelet releasate of eight patients with storage pool disease and bleeding symptoms to that of nine unrelated healthy controls (Di Michele et al. 2011). This more advanced technology identified 60 and 14 protein spots that varied in the technical and biological replicate studies, and most proteins are cytoskeleton-related. It was hypothesized that the dense granule defect in these storage pool disease cases might be due to an underlying cytoskeleton defect. A similar proteomic design was used to analyze platelet proteins from patients with dominant macrothrombocytopenia (Karmakar et al. 2015). Patients have altered levels of actin-binding proteins, peroxiredoxin 2, protein disulfide isomerase, and transthyretin that might be associated with the structural changes of their platelets.

Platelet Proteomics to Study Cardiovascular Disease

Arterial thrombosis is a pivotal event in the development of cardiovascular disease, and platelets play a fundamental role in this process. Platelet proteomics has been applied to study this complex disease, and we have selected some examples. The first study was performed in 2008 using 2DE and MS and focused on patients with arterial thrombosis, primarily with ischemic stroke (Arias-Salgado et al. 2008). Most of the differences detected between groups were related to cytoskeletal changes, which supported the idea of preactivated platelets. 2DE and MS analysis of platelets from patients with non-ST segment elevation acute coronary syndrome (ACS) revealed 22 differentially expressed proteins compared to matched cases with chronic ischemic cardiopathy (Parguiña et al. 2010). Most of these proteins are interconnected as part of the network related to cell assembly and morphology and are predicted to participate in platelet activation via $\alpha_{IIb}\beta_3$ or GPVI receptors. Platelet proteins isolated from ACS patients admitted within 24 h of chest pain were compared to these from patients with stable coronary ischemic disease (Lopez-Farre et al. 2011). Different proteins involved in cytoskeleton, glycolysis pathway, and cellular-related antioxidant system were altered in the acute phase of the coronary event as identified via 2DE and MS. The platelet proteome of ST-elevation myocardial infarction (STEMI) patients was compared to that of stable chronic ischemic cardiopathy (CAD) patients again using 2DE and MS (Parguina et al. 2011). This study found 42 differentially expressed proteins with altered major signaling pathways that include proteins related to integrin, integrin-linked kinase, and GPVI signaling. The analysis of a coronary thrombus itself by proteomics using different techniques as 2DE with MS, 1-DE with LS-MALDI MS/MS, and 1-DE with LTQ-Orbitrap identified a total of 708 proteins (Alonso-Orgaz et al. 2014). Some of these proteins were co-expressed with the platelet marker CD41 and pointed out a potential activation of a focal adhesion pathway in platelets during thrombus formation. Proteomics of microvesicles isolated from plasma of STEMI versus CAD patients was performed by DIGE and MS (Vélez et al. 2014). About 102 proteins were identified that correspond to 25 unique differentially expressed proteins that have been linked to inflammation, infarction, and thrombogenesis. The most recent proteomic study used isolated thrombi from STEMI patients during percutaneous coronary intervention at different time points after onset of pain (3 or 6 h) (Ramaiola et al. 2015). Thrombi at 3 h were platelet-rich, while at 6 h leukocyte infiltration was noticed, and proteomic differences between these time points were mainly related to changes in the cell cytoskeletal-associated proteome.

Antiplatelet therapies are widely used to prevent myocardial infarction, stroke, and other cardiovascular events. Platelet proteomics proved also to be useful to study the biological effects of antiplatelet therapy and to study mechanisms of patients' resistance to such drugs. The platelet proteome was analyzed using 2DE for patients with stable angina undergoing percutaneous coronary intervention before angiography, 12 h after clopidogrel, and 24 h after the intervention (Volpi et al. 2012). Protein changes were detected associated with platelet activation and clopidogrel response, and most proteins belong to the cytoskeleton rearrangement, energetic metabolism, and oxidative stress functional classes. Platelet protein expression profiles from aspirin (ASA)-resistant and ASA-sensitive CAD patients were compared using 2DE and MS (Mateos-Caceres et al. 2010). Differences were found for proteins involved in energetic metabolism, cytoskeleton, oxidative stress, and cell survival, which might be due to their different ability to respond to ASA. Pre- and post-aspirin treatment platelet lysate samples (300 mg daily for 28 days) from two ASA-resistant and four ASA-sensitive healthy subjects were analyzed by GeLC-MS/MS (Floyd et al. 2014). Though the

groups presented with no detectable changes in the platelet proteome at baseline, 406 differential proteins were present after aspirin treatment with a marked increase in GPIIIa for ASA resistance. Recently, a manually curated biochemical reaction network of platelet metabolism was constructed using 33 proteomic datasets and 354 literature references (Thomas et al. 2014). The effect of ASA resistance was evaluated using constraint-based modeling, providing evidence for a redirection of glycolytic, fatty acid, and nucleotide metabolism toward eicosanoid synthesis and reactive oxygen species stress as validated by novel proteomic data. The availability of such a network will stimulate data-driven system analysis of platelet metabolism in order to gain insights into pathologies.

Platelet Proteomics to Study Other Disorders

Platelet proteomics has also been used to gain insights in diseases that are linked to platelets, such as for understanding thrombosis risks for phosphomannomutase 2 (PMM2) deficiency, sepsis, deep vein thrombosis (DVT), diabetes and uremia, inflammation in cystic fibrosis, and bleeding in myelodysplastic syndrome (MDS). Some of these proteomic studies are discussed as examples. A subproteomic analysis was performed for patients with the congenital disorder of glycosylation PMM2 that have an increased risk for thrombosis (de la Morena-Barrio et al. 2014). DIGE analysis of the N-glycoproteins however showed no quantitative or qualitative differences between patients and controls. Platelet 2DE profiles of septic patients versus healthy controls resulted in five differentially expressed proteins that include GPIX and GPIIb (Liu et al. 2014). Proteins isolated form plasma-derived microparticles were tagged with iTRAQ reagents and analyzed by 2DE with LS-MALDI MS/MS for nine patients with DVT and six healthy controls (Ramacciotti et al. 2010). The differentially expressed or depleted proteins are expected to influence thrombosis via inflammation, cell shedding, inhibition of fibrinolysis, and hemostatic plug formation. Platelets from 13 diabetic patients were analyzed before and 12 weeks after pioglitazone therapy using DIGE and LS-MS/MS (Randriamboavonjy et al. 2012). More than half of the differentially expressed protein spots identified were known calpain substrates and could be classified as cytoskeletal proteins and signaling molecules. This study suggests that diabetes-induced platelet dysfunction might be due to calpain activation. Platelet proteomics was performed for uremia patients with functional versus dysfunctional platelets as tested by PFA100 (Marques et al. 2010). 2DE and MS analysis showed changes in protein levels that might have occurred at the megakaryocyte level. Shotgun nUPLC-MSE and 2DE were used to compare platelet proteins between cystic fibrosis (CF) patients and healthy controls (Pieroni et al. 2011). CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator that is expressed on platelets and related to changes in inflammation for which the proteomic study provided evidence of changes in integrin signaling. The platelet proteome of myelodysplastic syndrome (MDS) patients was analyzed with DIGE and showed lower levels of proteins that are important for integrin $\alpha_{IIb}\beta_3$ signaling (Fröbel et al. 2013). Impaired platelet aggregation might therefore explain the bleeding complications observed for MDS patients even at sufficient platelet counts.

Less obvious are the platelet proteomic studies that have been designed to unravel disease mechanisms for neuropathologies. However, it is known that platelets do share common characteristics with neurons (Goubau et al. 2013). Again, examples of proteomic studies conducted for Alzheimer's disease (AD), but also for monogenetic neurological diseases, are discussed. The platelet proteome was analyzed by DIGE for 34 AD cases, 13 cases with vascular dementia, 15 Parkinson cases, and 49 healthy controls (Zellner et al. 2012). The study suggested that Mao-B platelet protein levels could be a biomarker for age-related dementia in AD. In addition, the platelet membrane proteome was determined for five AD cases versus controls using 1-DE and LC-MS/MS (Donovan et al. 2013). A total of 144 proteins were altered that represent secretory granule proteins, and it was suggested that platelets may serve as a source of blood-based biomarkers in neuropathologies. DIGE with MS identification was used to analyze platelets and fibroblasts from patients with alternating lemiplegia of childhood (AHC) due to *ATP1A3* mutations (Di Michele et al. 2013). A total of 93 proteins have a different expression of which seven were detected in both cell types, and this included lysosomal protein cathepsin. Functional validation studies showed that AHC might be associated with a defective regulation of apoptosis via the lysosomal cathepsin pathway.

Conclusion

The application of the proteomics and subproteomic approaches described above has provided an invaluable contribution to the elucidation of platelet physiological mechanisms. We have included a figure with the most important items discussed in this chapter (Fig. 1). Moreover, they also provided remarkable insights into the molecular basis underlying platelet-related diseases, though several challenges still remain to be addressed. However, we are confident that the integration of the most recent advances in MS-based techniques in platelet proteomic studies will allow further delineation of the mechanisms implicated in diseases and identify new biomarkers or drug targets.

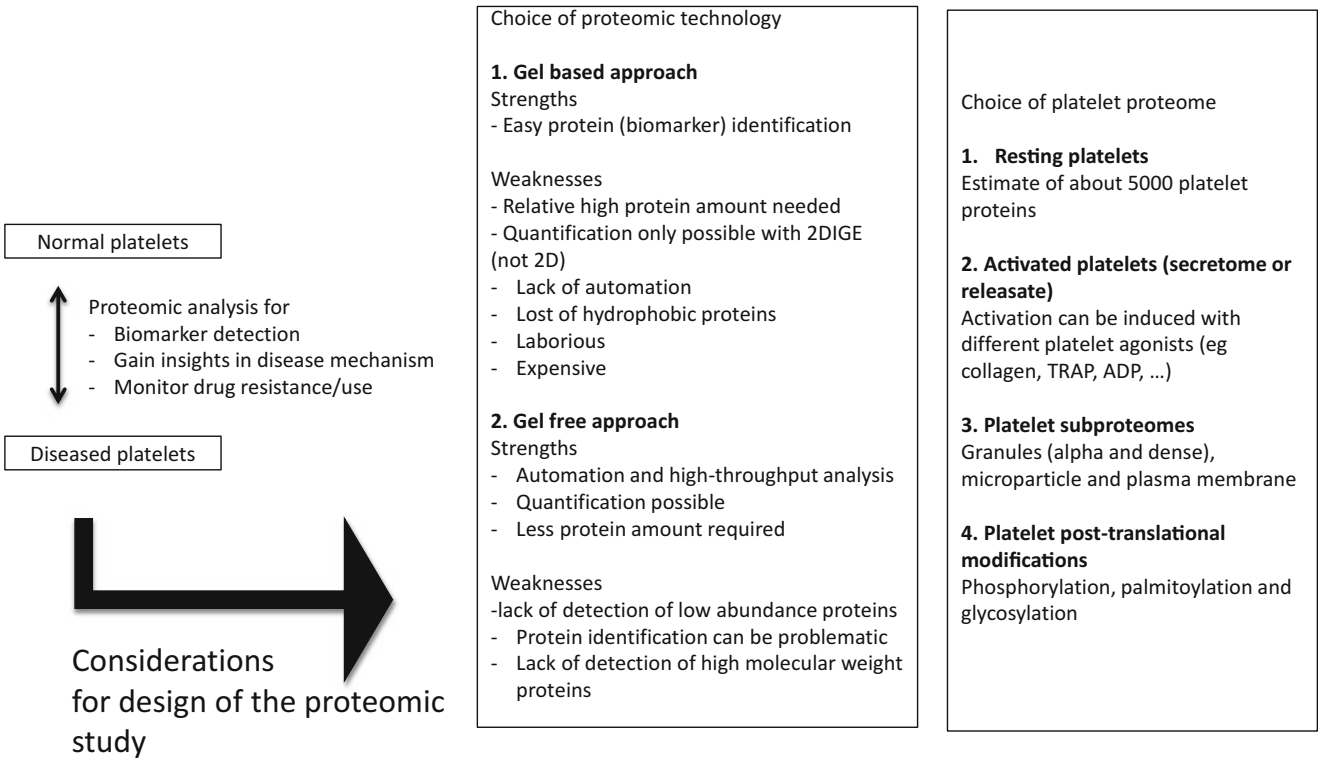

Fig. 1 Overview of proteomic technologies used in platelet research

Take-Home Messages

- Proteomics represents a powerful tool to study platelet proteins and the molecular mechanisms regulating unknown platelet functions and platelet-related diseases.
- Gel-based and gel-free proteomics technologies, both with their own advantages and limits, have been efficiently used to analyze platelets in physiological and pathological conditions.
- The experimental design of platelet proteomic studies should take into account the appropriate controls, technical/biological replicates, and sample preparation protocols.
- Proteomics can be applied to the study of (1) whole platelet proteome (in resting or activated condition); (2) platelet subproteomes (granules, microparticles, plasma membrane); and platelet PTMs (phosphoproteome, glycoproteome, palmitoylome).
- Platelet proteomics has been used to investigate platelet-related bleeding disorders and cardiovascular diseases, but also diseases less obviously linked to platelets.

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The Platelet PARs

Amal Arachiche and Marvin T. Nieman

Abstract

The serine protease thrombin is a potent activator of platelets. It binds to two classes of receptors, the GPIb-IX complex and protease-activated receptors (PARs). PARs constitute a family of four G protein-coupled receptors named PARs 1–4 that mediate protease signaling in a wide variety of cells. In this chapter we describe the genomic organization and expression of PARs in platelets from humans and other species. Thrombin is the primary activator of PARs in platelets. We focus on the factors that determine the specificity and rate of cleavage PARs by thrombin, which are the initiating events of thrombin-induced platelet activation. Human platelets express PAR1 and PAR4, which have both overlapping and distinct signaling pathways. These differences have become increasingly important as therapeutics targeting PAR1 and PAR4 are developed. In addition to thrombin-PAR interactions, the activation and downstream signaling of PAR subtypes is influenced by dimerization with one another and other platelet GPCRs. We also discuss the recent identification of genetic variations that impact PAR4 signaling in humans. Finally, we highlight the differences in PAR expression on platelets across species that impact how animal models can be used as preclinical tools.

Key Points

Thrombin signaling in human platelets is mediated by protease-activated receptor 1 (PAR1) and PAR4.

PARs are G protein-coupled receptors that are activated by proteolysis of their N-terminus to expose the tethered ligand.

PAR1 and PAR4 activate overlapping and independent signaling cascades in human platelets.

PARs form homo- and hetero-oligomers that affect their activation and signaling.

Polymorphisms in the PAR4 gene (f2rl3) result in sequence variants with different reactivities, which results in altered responses to PAR4 agonists.

The expression profile of PARs on platelets varies between species, which limits the clinical translation of some animal models.

Introduction

Protease-activated receptors are members of the G protein-coupled receptor (GPCR) superfamily that are widely expressed and signal through multiple G proteins. There are four members of the PAR family of receptors, PAR1, PAR2, PAR3, and PAR4. Here, we describe the expression and activation mechanism of PAR1, PAR3, and PAR4 by thrombin and their roles in platelet signaling. PAR2 is not expressed on platelets and is not discussed in this chapter. We also give a brief overview of the expression profile of PARs in platelet from other species that are frequently used as animal models.

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Historical Perspective

In the late 1980s, several laboratories focused their work to solve the enigma of how a serine protease, thrombin, activates platelets. Thrombin is the most potent platelet agonist and plays an important role in thrombus formation and hemostasis. It is generated in the plasma by the prothrombinase complex, which catalyzes the proteolytic activation of the zymogen, prothrombin (FII), to form the active protease, thrombin (FIIa) (Krishnaswamy 2013). Binding sites with three distinct characteristics have been identified for thrombin on human platelets: high-affinity binding sites ($K_d = 0.3$ nM; 50 sites/platelet), intermediate-affinity binding sites ($K_d = 10$ nM; 1700 sites/platelet), and low-affinity nonspecific binding sites ($K_d = 3$ nM; 600,000 sites/platelet) (Harmon and Jamieson 1985). The binding sites are specific to thrombin because prothrombin does not bind to platelets. Originally, two platelet receptors were proposed as potential receptors for thrombin, glycoprotein Ib (GPIb) and glycoprotein V (GPV). However, both GPV and GPIb receptors did not fully explain how platelets response to thrombin. First, platelets from patients with Bernard-Soulier syndrome (BSS), deficient in both GPIb and GPV, respond to thrombin the same as platelets from control individuals. Second, there is no correlation between the cleavage kinetics of GPV by thrombin and the kinetics of platelet activation (Clemetson and Clemetson 1995; Kahn et al. 1999a). Finally, in the early 1990s, protease-activated receptor 1 (PAR1) was identified as the main thrombin receptor on human platelets (Vu et al. 1991a). The cloning of PAR1 lead to the identification of the three other family members (PARs 2–4).

Protease-Activated Receptor 1 (PAR1)

Protease-activated receptor 1 was the first thrombin receptor cloned in 1991 (Vu et al. 1991a). The gene encoding PAR1 (*f2r*) is located in chromosome 5 (5q13). PAR1 has a genomic structure that contains two exons separated by an intron of approximately 15 kb (Kahn et al. 1998a). The *f2r* gene encodes for a 425 amino acid protein with seven transmembrane domains. The general activation mechanism common to all members of the PAR family were largely worked out for PAR1 (Fig. 1). The N-terminal domain of PAR1 is oriented to the extracellular space and contains the recognition site(s) for thrombin. Following cleavage by thrombin at arginine 41 (. . .$LDPR_{41}$/SFLLRN. . .), the new N-terminal exodomain (SFLLRNP. . .) acts as tethered ligand that binds to the second extracellular loop of PAR1. This leads to a conformational change in the receptor, which initiates signal transduction (Nanevicz et al. 1995). The synthetic peptide,

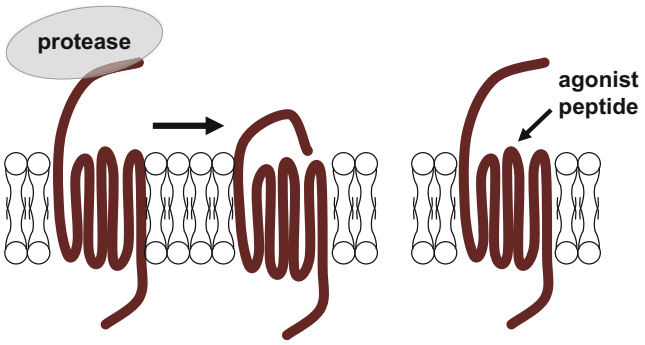

Fig. 1 A schematic of PAR activation by the tethered ligand. The protease binds and cleaves the N-terminal exodomain. The newly formed N-terminus binds to the extracellular loops to initiate downstream signaling events (*left panel*). Experimentally, PARs can be activated via agonist peptides that mimic the cleaved N-terminus (*right panel*). These are often referred to as TRAPs (thrombin receptor-activating peptides)

SFLLRN, mimics the first six amino acids of newly formed N-terminus and can activate PAR1 independent of cleavage (Gerszten et al. 1994). The PAR activation peptides, sometimes referred to as thrombin receptor-activating peptides (TRAPs), have been used experimentally to selectively activate PARs on cells to study the signaling events that are downstream to specific PAR family members. One caveat is that SFLLRN will also activate PAR2. The peptide TFLLRN should be used for cells co-expressing PAR1 and PAR2, such as endothelial cells.

The simplistic view of PAR activation by the tethered ligand is that the N-terminus bends over and activates the receptor by binding extracellular loop 2. However, NMR and mutagenesis studies with the PAR1 exodomain suggest that the N-terminus actually folds back on itself to form a secondary structure that is required for activation (Seeley et al. 2003). It is not known if this is a general mechanism of PAR activation as analogous studies have not been reported for other PAR family members. Although PAR1 was originally identified as the thrombin receptor, it is widely expressed and can be cleaved by multiple proteases to activate distinct signaling pathways through biased agonism (see discussion below) (Koukos et al. 2011; Zhao et al. 2014). It is not known how the alternative cleavage sites influence the secondary structure of the PAR1 N-terminus.

The unique activation mechanism of PARs is irreversible, which prohibits receptor recycling following activation. Following stimulation, PAR1 undergoes desensitization due to receptor internalization (Molino et al. 1997; Brass et al. 1992). A key regulatory step of PAR1 signaling is phosphorylation of the C-terminal tail (Shapiro et al. 2000). More recent studies have also linked PAR1 internalization to ubiquitination and glycosylation (Chen et al. 2011; Soto and Trejo 2010; Russo et al. 2009a; Wolfe et al. 2007).

Protease-Activated Receptor 3 (PAR3)

Protease-activated receptor 3 was cloned in 1997 as the second thrombin receptor and third member of the PAR family (Ishihara et al. 1997). The gene encoding PAR3, *f2rl2*, co-localizes with *f2r* (the PAR1 gene) at chromosome 5q13. It is located at 5′ of *f2r* and separated by less than 25 kb (Schmidt et al. 1996; Kahn et al. 1998a). The common locus of the genes for PAR1 (*f2r*), PAR2 (*f2rl1*), and PAR3 (*f2rl2*) suggests a common ancestral gene for this family of receptors. The genomic organization of *f2rl2* is similar to *f2r*, with one small exon and one large exon. In contrast to *f2r*, the two exons of *f2rl2* are separated by a small intron of approximately 4.5 kb (Kahn et al. 1998a).

The PAR3 mRNA encodes for a 373 amino acid protein, which shares 27 % amino acid sequence identity with PAR1. Similar to PAR1, the N-terminal exodomain of PAR3 has two thrombin recognition sites; the cleavage site is at lysine 38 (. . .LPIK$_{38}$/TFRGAP. . .) and a hirudin-like sequence (FEEFP) that binds thrombin's exosite I (Ishihara et al. 1997; Ayala et al. 2001; Bah et al. 2007). A major difference between PAR1 and PAR3 is that the C-terminal tail of PAR3 is significantly shorter than that of PAR1 (13 versus 51 amino acids). The shorter C-terminal tail likely affects the coupling of PAR3 to intracellular signaling machinery and subsequent signal transduction for platelet activation. PAR3-activating peptide, TFRGAP, does not activate human platelets, *Xenopus* oocytes, or COS7 cells transfected with human PAR3, suggesting that it does not have a signaling function on its own (Andersen et al. 1999; Ishihara et al. 1997). In human platelets, the expression level of PAR3 is substantially lower than PAR1, 150–200 copies versus 1500–2000 copies on the surface, respectively (Brass et al. 1992; Schmidt et al. 1998). It has been difficult to define a functional role for PAR3 on human platelets (Ishihara et al. 1997). In contrast, PAR3 is highly expressed on mouse platelets in which it regulates the sensitivity and response of PAR4 at both low and high thrombin concentrations (Nakanishi-Matsui et al. 2000; Arachiche et al. 2013a). Similar to human platelets, a direct signaling role for PAR3 has not been described.

Protease-Activated Receptor 4 (PAR4)

Protease-activated receptor 4 (PAR4) was the fourth member of PAR family and the third thrombin receptor identified (Xu et al. 1998; Kahn et al. 1998b). In contrast to *f2r* and *f2rl2*, the gene encoding PAR4, *f2rl3*, maps to chromosome 19p12. The *f2rl3* gene is also organized into two exons, but the intron separating the two exons is small (~0.25 kb) compared to other PARs (Kahn et al. 1998a). The expression of PAR4 mRNA was detected in platelets and a number of other human tissues such as the lung, pancreas, thyroid, testis, and small intestine. PAR4 expression was not detected in the brain, kidney, spinal cord, and peripheral blood leukocytes (Kahn et al. 1999b; Xu et al. 1998).

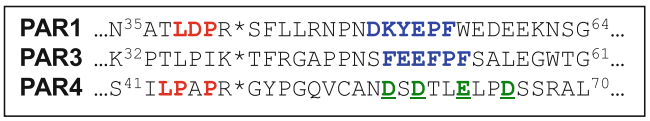

Fig. 2 Schematic diagram of PAR1, PAR3, and PAR4 exodomains. PAR1 and PAR3 primary sequence with amino acids that interact with the thrombin cleavage site highlighted in *red* and the hirudin-like sequence highlighted in *blue*. PAR4 primary sequence with amino acids that interact with the thrombin cleavage site highlighted in *red* and the anionic cluster highlighted in *green* and *underlined*

PAR4 is a protein that is 385 amino acids in length with 27 % and 30 % amino acid sequence identity with PAR1 and PAR3, respectively (Xu et al. 1998). Thrombin activates PAR4 by the cleavage of the N-terminal of PAR4 at arginine 47 (. . .LPAPR$_{47}$/GYPGQV. . .) (Fig. 2). The synthetic peptide (GYPGQV) corresponding to the unmasked amino terminus of PAR4 also activates the receptor. However, peptide library screens demonstrated that the peptide AYPGKF is more potent and is commonly used experimentally (Hollenberg and Saifeddine 2001).

PAR1 has been more extensively studied than PAR4, which has lead to the development of the FDA-approved drug vorapaxar (Baker et al. 2014). However, recently it has been recognized that PAR4 has unique signaling properties that may make PAR4 an attractive target for therapeutics (Kuliopulos and Covic 2003; Young et al. 2013; Mumaw et al. 2014). Further, the identification of PAR4 sequence variants that result in receptors with different reactivities highlights the importance that each receptor contributes to thrombin signaling. Finally, since PAR1 is not expressed on mouse platelets, signaling ascribed to PAR4 in mouse studies need to be interpreted with caution. For example, the C-terminus of mouse PAR4 has properties similar to human PAR1, which affects binding of some antagonists (Aisiku et al. 2015). These differences are discussed below.

Activation of PARs by Thrombin

Much effort has been made to characterize the molecular mechanisms by which thrombin interacts with its substrates. In general, thrombin's interaction with its substrates consists of three parts: (1) it binds via its anionic binding exosite I to a site on some of its substrates termed a "hirudin-like sequence," (2) it binds through amino acids that surround the active site to some amino acids at the P5-P2 positions on the substrate, and (3) thrombin's active site interacts

with the substrate P1-P1′ position. The rate of cleavage of PARs by the activating protease is the rate-limiting step for signaling.

In addition to binding thrombin's active site, PAR1 has a hirudin-like sequence ($D^{50}KYPEK^{55}$) that binds thrombin's exosite I which induces allosteric effects on thrombin, lowering the energy required for PAR1 cleavage (Fig. 2) (Liu et al. 1991; Ayala et al. 2001; Jacques et al. 2000). The importance of the hirudin-like sequence has been confirmed with thrombin exosite mutations as well as PAR1 exodomain mutations (Ayala et al. 2001; Jacques et al. 2000; Vu et al. 1991b; Myles et al. 2001). Further, mutations around the thrombin cleavage site do not dramatically affect the K_m of thrombin cleaving PAR1 due to the exosite I binding region. However, the k_{cat} is reduced sevenfold when Leu^{38} at P4 is mutated to alanine and twofold when Pro^{40} at P2 is mutated (Fig. 2) (Nieman and Schmaier 2007). When PAR1 peptides were co-crystallized with thrombin, structures were solved in which the active site of thrombin interacted with $L^{38}DPR^{41}$ or the exosite I region interacted with $K^{51}YEPF^{55}$. However, none of these structures had the exosite I and the active site simultaneously filled (Mathews et al. 1994). The hirudin-like sequence ($K^{51}YEPF^{55}$) induces a change of conformation when the Ala^{190}-Gly^{197} region of thrombin with the Glu^{192} side chain becomes disordered, helping to accommodate the negatively charged Asp at P3 for PAR1. The crystals in which the active site is filled by the $L^{38}DPR^{41}$ sequence shows that Leu^{38} occupies the aryl-binding pocket formed by Ile^{174} and Trp^{215} as predicted by Bode et al. (1992). The intervening sequence between $L^{38}DPR^{41}$ and $K^{51}YEPF^{55}$ of PAR1 ($F^{43}LLRNP^{48}$) is disordered with no electron density (Mathews et al. 1994). Using the data from the two sets of crystals in Mathews et al. (PDB ID codes 1NRS and 1NRN), Huntington has proposed models for a single PAR1 peptide interacting with thrombin's active site and exosite I simultaneously, which has largely been confirmed by more recent structural studies (Huntington 2005; Gandhi et al. 2010).

PAR4 does not have a hirudin-like sequence (Fig. 2) (Xu et al. 1998). Based on studies with peptides and recombinant exodomains, the primary sites of PAR4 interaction with α-thrombin is at the thrombin cleavage site. In particular, amino acids Leu^{43} at P5, Pro^{44} at P4, and Pro^{46} at P2 are important α-thrombin interaction sites (Cleary et al. 2002; Jacques and Kuliopulos 2003; Nieman and Schmaier 2007). However, individual point mutations at Leu^{43}, Pro^{44}, or Pro^{46} do not influence thrombin binding (i.e., did not influence the K_m) but did reduce the rate of cleavage indicating that Leu^{43}, Pro^{44}, or Pro^{46} are important for orienting PAR4 in the active site of thrombin for efficient cleavage (Nieman and Schmaier 2007). More importantly, these data also suggest that, like PAR1, PAR4 has extended contacts with α-thrombin that minimize the influence of the individual amino acids at the cleavage site (Fig. 1) (Nieman and Schmaier 2007). Earlier work by Jacques and Kuliopulos, using purified exodomains, demonstrates that mutations of the anionic cluster in the PAR4 exodomain (Asp^{57}, Asp^{59}, Glu^{62}, Asp^{65}) (see Fig. 2) decreased the K_m of thrombin binding fourfold from 56 to 208 nM (Jacques and Kuliopulos 2003). Further experiments demonstrate that the anionic cluster stabilizes the interaction with thrombin by slowing the dissociation rate (Jacques and Kuliopulos 2003). These functional data are supported by structural and modeling studies. Ayala et al. showed via molecular modeling using peptides that Leu^{43} may be important for interaction with thrombin residues Leu^{99}, Ile^{174}, and Trp^{215} (Ayala et al. 2001). However, NMR studies with PAR4 peptides by Cleary et al. demonstrate that this leucine is flexible and can interact with thrombin as well as Pro^{44} or Pro^{46} to stabilize secondary structure at the thrombin cleavage site of PAR4 (Cleary et al. 2002). Crystallography studies show that PAR4 interacts with thrombin's gamma (autolysis) loop (Bah et al. 2007).

PAR4 is not an efficient thrombin substrate when expressed on cells alone and requires approximately tenfold more thrombin for activation (Jacques and Kuliopulos 2003; Nieman 2008). However, when PAR4 is co-expressed with PAR1 in heterologous systems as it is on human platelets, the rate of PAR4 cleavage is enhanced six- to tenfold (Jacques and Kuliopulos 2003; Nieman 2008; Arachiche et al. 2013b). The enhanced rate of PAR4 cleavage is similar that first described for the functional interaction between PAR3 and PAR4 on mouse platelets (Nakanishi-Matsui et al. 2000). In this model, thrombin remains bound to the hirudin-like sequence following proteolysis at the cleavage site, which enhances the activation of an adjacent PAR4 (Fig. 3). There are two potential mechanisms for this model. First, the higher-affinity binding site on PAR1 or PAR3 (the hirudin-like sequence) may increase the local concentration of thrombin near the platelet surface to facilitate the activation of PAR4. Alternatively, based on biochemical and structural data with other tight exosite I binders, PAR1's hirudin-like sequence may induce thrombin into the protease conformation with an open active site to facilitate PAR4 cleavage (Huntington 2012; Kamath et al. 2010). The latter model is also supported by structural models of murine thrombin bound to peptides from PAR3 and PAR4 (Bah et al. 2007).

Thrombin Signaling in Human Platelets

The responses of platelets to thrombin stimulation are many and varied. PAR1 and PAR4 cooperate to mediate the full range of thrombin signaling in human platelets by coupling to multiple heterotrimeric G proteins (Kahn et al. 1999b).

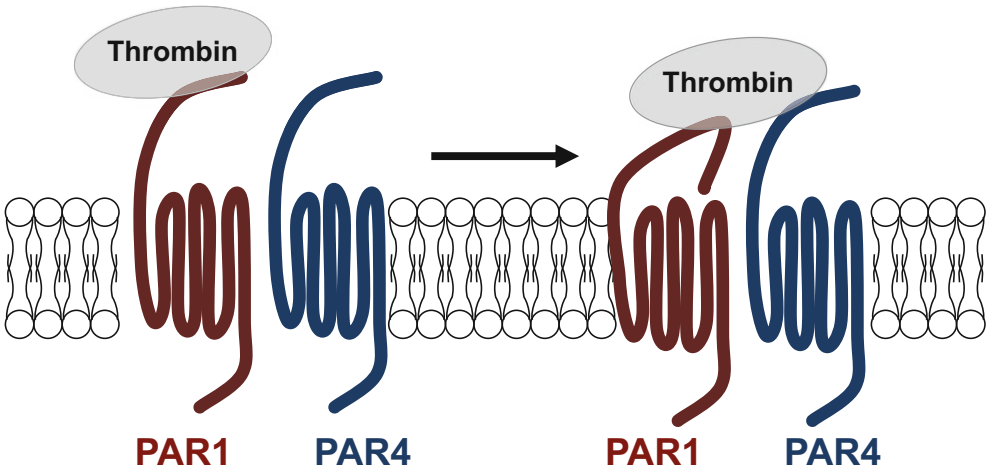

Fig. 3 PAR1 serves as a cofactor for PAR4 activation by thrombin on human platelets. The rate of PAR4 cleavage is enhanced by co-expression of PAR1 (human platelets) or PAR3 (mouse platelets) via the hirudin-like sequence; see text for details

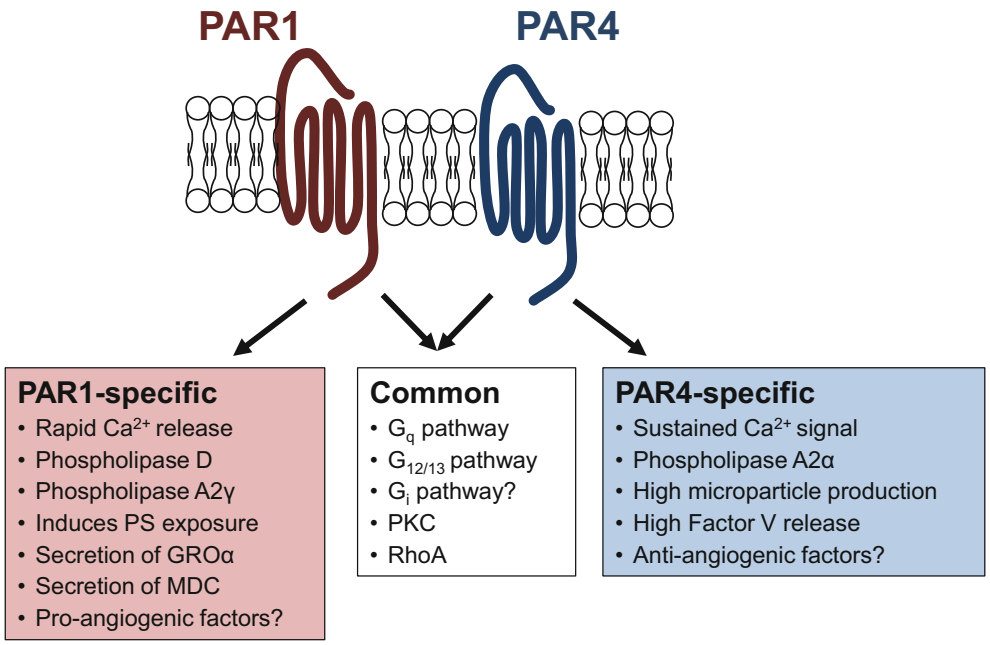

Fig. 4 PAR1 and PAR4 have overlapping and distinct downstream signaling events. PAR1 and PAR4 both signal through multiple G proteins; however, the duration and kinetics of signaling can differ

PAR1 and PAR4 have several overlapping signaling functions, which led to the original hypothesis that PAR4 is a redundant, backup receptor. Several studies have since shown these receptors can activate unique signaling pathways and, in some cases, the same pathways with distinct kinetics (Fig. 4).

Overlapping Signaling Between PAR1 and PAR4

In human platelets, PAR1 and PAR4 both transduce signals through $G\alpha_q$ and $G\alpha_{12/13}$. The activation of $G\alpha_q$ stimulates the formation of inositol triphosphate (IP3) and diacylglycerol (DAG), which induces intracellular calcium mobilization and protein kinase C (PKC) activation, respectively (Hung et al. 1992; Offermanns et al. 1997). This pathway controls a variety of platelet responses including granule secretion, integrin activation, and platelet aggregation in platelets. The activation of $G\alpha_{12/13}$ mediates Rho guanine nucleotide exchange factors and RhoA signaling pathways, which controls platelet shape change (Moers et al. 2003; Huang et al. 2007). $G\alpha_i$ signaling in platelets mediates inhibition of adenylate cyclase activity but also induces platelet shape change, secretion, and calcium mobilization. The direct interaction between PARs and G_i in human platelets is controversial. PAR1 directly couples to G_i in COS7 cells transfected with PAR1 (McCoy et al. 2012) and, in some cases, platelets (Voss et al. 2007). In other studies, PAR1 and PAR4 did not couple directly to G_i. Here, the G_i pathway was mediated by secondary release of ADP, which acts on the G_i-coupled ADP receptor, P2Y12 (Jantzen et al. 2001; Kim et al. 2002, 2006).

Calcium Mobilization

The most dramatic differences between PAR1 and PAR4 signaling in platelets is the kinetics and duration of intracellular Ca^{2+} signaling (Covic et al. 2000; Vaidyula and Rao 2003). Prior to the identification of PAR4, it was recognized that Ca^{2+} mobilization in thrombin-stimulated platelets was different from that of PAR1 agonist peptide-stimulated platelets. Further studies revealed that the PAR1 agonist peptide is a partial agonist of human platelets (Lau et al. 1994). The initial increase in intracellular Ca^{2+} was the same for thrombin and the agonist peptide. In contrast, when platelet was stimulated with the PAR1 agonist peptide in the absence of extracellular Ca^{2+}, the Ca^{2+} flux returned to baseline levels faster and induced a reduced level of lysosome release. Finally, the overall magnitude and duration of the Ca^{2+} signal was greater in thrombin-stimulated platelets (Heemskerk et al. 1997). The identification of PAR4 reconciled these differences when it was demonstrated that there is a distinct wave of Ca^{2+} signaling from PAR4 (Covic et al. 2000). The prolonged Ca^{2+} stimulus associated appears to be required for stable clot formation and full spreading on fibrinogen in response to thrombin in a p38- and ERK1/2-dependent manner (Covic et al. 2002; Mazharian et al. 2007). The proposed mechanism for sustained signaling from PAR4 is that PAR4 is internalized more slowly compared to PAR1. In cultured fibroblast, 50 % of PAR1 is internalized following stimulation compared to 20 % of PAR4 (Shapiro et al. 2000).

Cooperation Between Thrombin and ADP Signaling

PAR1 and PAR4 also synergize with ADP signaling in platelets. One study has shown that PAR4, but not PAR1 signaling to Akt, is dependent on P2Y12, particularly under conditions of limited calcium concentration (Holinstat et al. 2006). A number of other reports have found results suggesting that PAR1 synergizes independently with P2Y12, rather than PAR4 (Resendiz et al. 2007; Wu et al. 2010; Jiang et al. 2013). PAR1 but not PAR4 directly influenced ADP-induced platelet granule secretion and second wave of aggregation. Blocking PAR1 activation by SCH79797 abolished the ATP secretion, $\alpha_{IIb}\beta_3$ activation, P-selectin expression, and the second wave of platelet aggregation associated with partial disaggregation. In contrast, PAR4 antagonist tcY-NH2 had no effect on these responses (Jiang et al. 2013). Furthermore, selective activation of PAR1 by SFLLRN together with collagen enhanced the increase in exposure of procoagulant phosphatidylserine (PS) exposure on the surface of platelets, as simultaneous stimulation of platelets with thrombin and collagen. The selective activation of PAR4 by the agonist peptide, GYPGQV, resulted in less PS exposure on the platelet surface (Andersen et al. 1999). In addition, stimulating platelets with ADP prior to SFLLRN produced a much greater increase in subpopulation of platelets that are PS positive compared to simultaneous stimulation of PAR1/P2Y12 (Shakhidzhanov et al. 2015). In sum, these studies indicate that P2Y12 and PAR1 signaling work in cooperation. Finally, in addition to the interaction of downstream signaling pathways, P2Y12 and PAR4 have a direct physical interaction that influences arrestin recruitment (see below) (Khan et al. 2014; Li et al. 2011).

Membrane Lipids and PAR1–PAR4 Downstream Signaling

PAR1 and PAR4 differentially regulate membrane lipid signaling. The role of sphingolipids has been demonstrated in both platelet function and platelet production (Shrimpton et al. 2002; Zhang et al. 2012b). Sphingomyelin (SM) is one of the major sphingolipids present in the plasma membrane and is hydrolyzed by sphingomyelinase (SMase) enzyme into ceramide and phosphorylcholine. Two types of SMase have been identified in platelets, the lysosomal phosphodiesterase acid SMase (A-SMase) and the membrane-bound neutral SMase (N-SMase); both enzymes regulate mouse and human platelets (Munzer et al. 2014; Chen et al. 2013). Thrombin or PAR4 agonist peptide, but not PAR1 agonist peptide, results in increased association of N-SMase with PAR4 in human platelets. The activation of N-SMase induced the generation of ceramide, which acts as a second messenger to induce the activation of the p38-MAPK-NF-kB signaling pathway in platelets (Chen et al. 2013). Thus far, the role of PAR1 or PAR4 in regulating acid sphingomyelinase (A-SMase) has not been demonstrated.

Arachidonic acid (AA) is another lipid membrane, which plays an important role in platelet function. AA is liberated form glycerophospholipid (GPL) via the action of phospholipase A2 (PLA2) and transformed to thromboxane A2 (TXA2) by sequential action of cyclooxygenase-1 (COX-1) and TXA2 synthase. Thrombin stimulates the generation of thromboxane A2 (TXA2), which, in turn, activates platelets via the thromboxane receptor (TP) and amplifies platelet activation to cause irreversible aggregation. PAR4 stimulation results in significantly greater TXA2 generation compared to PAR1 (Holinstat et al. 2011). Platelets express several PLA_2 isoform, including Ca^{2+}-sensitive 85 kDa cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), Ca^{2+}-insensitive 14 kDa secretory PLA_2 ($sPLA_2$), and Ca^{2+}-independent PLA2 ($iPLA_2\gamma$). It has been shown that thrombin-induced AA production in human platelets is dependent on $cPLA_2\alpha$ but not $sPLA_2$ (Bartoli et al. 1994).

The platelets from cPLA2α-deficient mice or patient with inherited deficiency of cPLA2α present a decrease in eicosanoid biosynthesis, such as prostaglandins, thromboxanes, and leukotrienes, which alters platelet function (Adler et al. 2008). In addition, mice lacking iPLA2γ present with a prolonged bleeding time and are protected from pulmonary thromboembolism (Yoda et al. 2014). In very recent work, a specific inhibitor of cPLA2α, giripladib, selectively inhibited PAR4 but not PAR1-mediated P-selectin expression in human platelets. However, specific inhibition of iPLA2γ with bromoenol lactone (BEL) significantly reduced P-selectin expression after PAR1, but not PAR4 activation in human platelets (Duvernay et al. 2015). Studies with human platelets show that inhibition of the phosphatidylcholine (PC)-derived phosphatidic acid (PA) formation by phospholipase D (PLD) inhibits platelet activation by PAR1-activating peptide. Thrombin or PAR4-activating peptides are insensitive to this inhibition. Furthermore, PAR1 but not PAR4 signals through phosphoinositide 3-kinase (PI3K) to activate integrin $\alpha_{IIb}\beta_3$ and induce platelet aggregation (Holinstat et al. 2007; Voss et al. 2007).

Granule Secretion and PAR1–PAR4 Downstream Signaling

Human platelets contain three types of storage granules, α-granules, dense granules, and lysosomes. The presence of distinct of α-granules with either pro-angiogenic or anti-angiogenic factors is controversial with studies supporting both selective and random secretion of granule contents. The differential release of pro-angiogenic and anti-angiogenic factors from platelets stimulated with PAR1 versus PAR4, respectively, was first described by Ma and colleagues (2005). The PAR4 agonist peptide stimulated the release of endostatin but suppressed the release of VEGF. In contrast, the PAR1 agonist peptide stimulated the release of VEGF and suppressed the release of endostatin. Curiously, stimulation with thrombin did not release either factor. Other studies also support a differential release of anti-angiogenic versus pro-angiogenic factors that are not due solely to either differential signal strength or kinetics of the respective agonists (Italiano et al. 2008; Chatterjee et al. 2011). Notably, PAR4-induced secretion of SDF-1 and endostatin were PI3K and Akt dependent, while PAR1-induced SDF-1 secretion was not (Chatterjee et al. 2011). PAR1 and PAR4 can also influence the degree of secretion. For example, PAR4 stimulation with the agonist peptide, AYPGKF, enhanced the surface expression of Factor V (1.6-fold) and P-selectin (0.8-fold) compared to PAR1 activation with SFLLRN (Duvernay et al. 2013). PAR4 activation also induced a threefold greater production of platelet microparticles compared with PAR1 activation. The RhoA pathway inhibitor Y-27632 reduced Factor V translocation and microparticle release downstream PAR4 stimulation to levels observed with PAR1 stimulation. These data indicate that PAR4 is mediating these events through the $G_{12/13}$-RhoA signaling axis. PAR4 activation also releases more CD40L from α-granules compared to PAR1. Conversely, PAR1 activation releases more growth-regulating oncogene-α (GRO-α) and macrophage-derived chemokine (MDC), compared to PAR4 (Nguyen et al. 2015).

In contrast, several studies have demonstrated random distribution of proteins within granules and that PAR1 and PAR4 stimulation leads to random release of granule contents from human platelets. A detailed analysis of α-granule proteins with quantitative immunofluorescence co-localization with pair-wise comparisons demonstrates the presence of one type of α-granule with random packing (Kamykowski et al. 2011). An analysis of the rates of secretion of several granules shows that the differences in α-granule release observed between PAR1 and PAR4 are based on the kinetics of granule release due to the strength of the agonist (Jonnalagadda et al. 2012). Furthermore, the RhoA activation downstream $G_{12/13}$ and G_q induced the same level of dense granule release in response to PAR1 or PAR4 activation (Jin et al. 2009). Recent work from van Holten et al. using mass spectrometry (MS)-based quantitative proteomic analysis and enzyme-linked immunosorbent assay (ELISA) shows that PAR1 or PAR4 activation of platelets results in the same α-granule release (van Holten et al. 2014). Finally, it is difficult to conclude if PAR1 and PAR4 induced similar or different protein mobilization, because the controversial results obtained from these studies might be due to the various techniques used to analyze platelet release, differences in agonist concentration, or difference in the number of human platelet samples analyzed.

Biased Signaling of PARs

GPCRs can signal through G protein and arrestin pathways. Biased agonists are those that preferentially activate specific pathways downstream of the receptor (Urban et al. 2007). In contrast, neutral agonists do not discriminate which pathways are activated. Biased signaling downstream of PARs can be mediated by cofactors, alternative cleavage sites, or pharmacologically (Zhao et al. 2014; Lin et al. 2013; Aisiku et al. 2015; Dowal et al. 2011). Studies aimed at understanding and directing the multitude of signaling events have largely been focused in endothelial cells. In this review we will focus on biased signaling as it relates to platelets.

Biased Signaling from PARs in Platelets

Thrombin is a neutral agonist for PAR1 and does not discriminate the downstream pathways that are activated. The first description of a PAR1 ligand that demonstrated specific signaling events in platelets was the activation peptide YFLLRNP (Rasmussen et al. 1993; Bauer et al. 1999). At low concentrations, this version of the PAR1 activation peptide induces shape change through $G_{12/13}$ but does not mediate G_q signaling and, as a result, does not induce full aggregation. Observations such as these opened the possibility of targeting specific pathways downstream of PARs. Since PAR1 activation has many, and sometimes divergent, cellular responses, inhibiting all downstream signaling with an orthosteric inhibitor such as vorapaxar (Zontivity) may not be ideal. An alternative approach that has be pursued by Flaumenhaft and colleagues is to screen for allosteric modulators of PAR1 that spare the cytoprotective signaling of PAR1 while blocking pathways that are detrimental to cell survival (Aisiku et al. 2015; Dowal et al. 2011). Their primary focus has been on PAR1 signaling in endothelial cell; however, the parmodulin compounds also selectively block G_q signaling in platelets. This family of compounds binds to the eighth helix on the cytoplasmic face of the receptor where they alter the interactions between the receptor and the G-alpha subunits.

The traditional role of arrestins is to regulate GPCR trafficking. The reports of arrestin function in platelets have been less straightforward. The internalization of P2Y receptors in an arrestin-dependent manner results in shutting down signaling (Nisar et al. 2012, 2011). In contrast, other reports suggest a direct signaling function of arrestin that promotes platelet activation (Schaff et al. 2012; Li et al. 2011). The specific contributions of arrestins to PAR signaling have been limited to PAR4 (Khan et al. 2014; Li et al. 2011). Recruitment of arrestin-2 to PAR4 is mediated by PAR4-P2Y12 heterodimerization where it has a positive signaling role. In mice that have arrestin-2 deleted, the platelet function is enhanced, and the time to thrombosis is shortened due to increased Src family kinase activation. These data support a direct signaling role for arrestin-2 rather than the expected desensitization of GPCR-mediated signaling that is expected.

Activation PARs by Proteases Other Than Thrombin

In addition to thrombin, several other serine proteases are capable of activating PARs such as factor Xa, plasmin, matrix metalloproteinases 1 and 13, elastase, activated protein C (APC), proteinase-3, granzyme, cathepsin G, and calpain (Zhao et al. 2014). Depending the cleavage site, PARs can be activated or inactivated by these proteases. Further, the alterative cleavage sites can generate novel tethered ligands that initiate specific signaling pathways. The panel of proteases activating PARs has been described for a variety of cell types. Here, we will focus on protease cleavage of PAR1 and PAR4 in the context of platelet function. The reader is directed to recent comprehensive reviews for other contexts (Russo et al. 2009b; Hollenberg et al. 2014; Zhao et al. 2014).

Factor Xa is directly upstream of thrombin in the coagulation cascade and can directly activate PARs (Camerer et al. 2002, 2000; Ruf et al. 2003). Early studies by Sinha et al. show that the pretreatment of platelet-rich plasma with FXa inhibited thrombin-induced platelet aggregation and TXA2 generation (Sinha et al. 1983). One potential caveat is distinguishing between FXa activation of prothrombin (which can subsequently activate PAR1) from direct activation of PAR1 by FXa. However, if the thrombin concentration is increased tenfold over FXa or if FVa is blocked on the surface of platelets using a specific anti-FVa antibody, the inhibition of TXA2 synthesis and platelet aggregation induced by FXa is reversed.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that are secreted as zymogens and, upon activation, degrade extracellular matrix proteins during tissue repair and cancer invasion (Woessner 1999). Matrix metalloproteinase-1 (MMP-1) directly activates platelets via PAR1. Stimulation of platelets with collagen results in the conversion of the inactive proMMP-1 to active MMP-1. Once activated, MMP-1 is capable of inducing platelet signaling by direct cleavage of PAR1 at a noncanonical site, Asp^{39} ($TLD_{39}PR_{41}SFLLRN$), that is distinct from canonical cleavage site of thrombin at Arg^{41} (Trivedi et al. 2009; Austin et al. 2013). In human platelets, the cleavage of PAR1 at Asp^{39} by MMP-1 induces $G_{12/13}$-Rho, p38 MAPK pathways, and shape change. However, intracellular calcium mobilization and platelet aggregation in response to MMP-1 are less potent than with thrombin (Austin et al. 2013; Trivedi et al. 2009). It should be noted that other cleavage sites on PAR1 have been reported for MMP-1 (Boire et al. 2005; Nesi and Fragai 2007).

The fibrinolytic enzyme plasmin cleaves both PAR1 and PAR4 on platelets. Four cleavage sites have been identified on the PAR1 exodomain (Arg^{41}, Arg^{70}, Lys^{76}, and Lys^{82}) (Kuliopulos et al. 1999). Kuliopulos and colleagues showed that although plasmin is capable of cleaving PAR1 at the canonical thrombin site, the predominant result is inactivation of PAR1 by truncating the tethered ligand at the distal sites. In the presence of a PAR1 inhibitor, plasmin stimulates platelet aggregation and shape change in human platelets via PAR4. Plasmin cleaves PAR4 at canonical thrombin cleavage site Arg^{47} (. . .$LPAPR_{47}$GYPGQV. . .) to generate PAR4 tethered ligand peptide (GYPGQV) (Quinton et al. 2004).

Cathepsin G is a serine protease found in the dense granules of neutrophils and is secreted upon neutrophil activation that cleaves both PAR1 and PAR4. The analysis of the N-terminal exodomain of PAR1 identified three potential cleavage site for cathepsin G: ($TLDPR_{41}SF_{43}LLRN...F_{55}..$). Preincubation of platelets with cathepsin G completely abolish thrombin-induced calcium mobilization (Parry et al. 1996). This is due to cathepsin G cleavage at Phe^{55} which leads to a loss of the PAR1 tethered ligand. Cathepsin G also cleaves PAR4 and induces calcium mobilization in human platelets and in PAR4 transfected fibroblasts (Sambrano et al. 2000). The story in mouse platelets is different. Cathepsin G blocks signaling by low thrombin concentrations by cleaving PAR3 and preventing it from acting as a cofactor for PAR4 (Cumashi et al. 2001). Cumashi and colleagues also showed that cathepsin G does not activate mouse PAR4.

Physical Interactions Between PARs

The molecular organization of GPCRs within the plasma membrane is controversial (Vischer et al. 2015). Homo- and heterodimers of GPCRs may influence signaling and can be thought of as allosteric modulators (Milligan and Smith 2007). The best evidence for a functional interaction between PAR homodimers on platelets is the dominant negative effect of PAR4 sequence variants that have low reactivity, which corroborates molecular studies (Edelstein et al. 2014; de la Fuente et al. 2012). Physiologically, there are examples of PAR heterodimers influencing the rate of activation, downstream signaling, and trafficking of the receptors in a diverse set of environments including platelets, smooth muscle cells, endothelial cells, and podocytes (Fig. 5) (Lin et al. 2013). As discussed above, the coordinated activity between PARs was first described for PAR3 and PAR4 on mouse platelets where PAR3 serves as a cofactor to enhance the rate of PAR4 cleavage by thrombin by approximately tenfold (Nakanishi-Matsui et al. 2000). An analogous mechanism was later demonstrated for PAR1 and PAR4 on human platelets (Leger et al. 2006; Nieman 2008). Each of these cases are dependent on PAR4 heterodimerization with PAR1 (human) or PAR3 (mouse) (Leger et al. 2006; Arachiche et al. 2013a, b).

PAR4, but not PAR1, also forms agonist-dependent heterodimers with another platelet GPCR, the ADP receptor P2Y12 (Li et al. 2011; Khan et al. 2014). The interaction between P2Y12 and PAR4 enhances the recruitment of β-arrestin-2 to PAR4 and mediates sustained signaling through Akt in human platelets. The PAR4-P2Y12 dimerization appears to be important to stabilize platelet plug formation. The interaction interface of the PAR4-P2Y12 heterodimer has been mapped to transmembrane helix 4 of PAR4. A mutation in PAR4 at the heterodimer interface disrupted the interaction with P2Y12 and prevents β-arrestin-2 recruitment and Akt activation (Khan et al. 2014). These data indicate that P2Y12 and PAR1 are competing with each other to interact with PAR4 as both receptors share the same heterodimer interface with PAR4 as well as the PAR4 homodimer interface (de la Fuente et al. 2012; Arachiche et al. 2013b; Khan et al. 2014). These interactions have the potential to not only influence platelet signaling but also how patients respond to therapies (Fig. 5) (Mumaw and Nieman 2014).

In addition to enhancing PAR4 activation, PAR3 can influence PAR4 signaling. PAR3-deficient mice have a 1.6-fold increase in the maximum Ca^{2+} mobilization and an increase in PKC activation but no effect on RhoA-GTP activation compared to platelets from wild-type mice. These results demonstrate that PAR3 regulates PAR4/G_q signaling pathway via a direct interaction with PAR4 indicating that dimerization of PARs may regulate coupling of G proteins to the receptor to influence downstream signaling. In addition to platelets, PAR3 also regulates PAR1 signaling in endothelial cells (McLaughlin et al. 2007; Stavenuiter and Mosnier 2014; Burnier and Mosnier 2013).

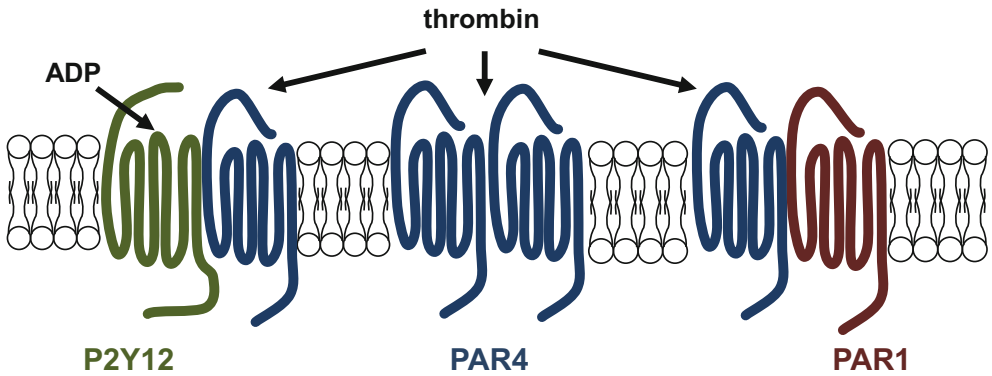

Fig. 5 The physical interaction between PARs. PARs for homo- and hetero-oligomers between PAR family members and other platelet GPCRs. These interactions influence both the rate of activation and signaling. Oligomerization also has the potential to influence the response to therapies. Note: PAR1 and P2Y12 also form homo-oligomers but are not shown in the figure for clarity

Polymorphisms and Sequence Variants

Single-nucleotide polymorphisms have been described for both PAR1 and PAR4. The challenge with SNPs is establishing a direct link from the identified polymorphisms to receptor expression or function and ultimately to a physiological output. One of the first described was a PAR1 polymorphism in an intron that affects PAR1 density on platelets and decreased platelet response to PAR1 agonists in individuals (Dupont et al. 2003). However, in a recent clinical study with 660 patients who underwent percutaneous coronary intervention (PCI), there was no evidence of increased major adverse cardiovascular events (MACE) or bleeding risk correlated with the polymorphism (Friedman et al. 2015).

The heritable interindividual variation in platelet reactivity has been directly linked to PAR4 (Bray et al. 2007; Edelstein et al. 2013, 2014; Tourdot et al. 2014). The Platelet RNA And eXpression 1 (PRAX1) study was designed to examine mRNAs and microRNAs associated with this difference in 154 healthy individuals who self-identify as black or white. In this population, Edelstein et al. showed that the black individuals had increased platelet response to PAR4 stimulation, higher expression of phosphatidylcholine transfer protein (PC-TP), and lower levels of miR-376c (Edelstein et al. 2013). The opposite was observed in the white individuals. Other platelet agonists, including PAR1, were not different between the groups. A second study identified two additional polymorphisms that change amino acids in PAR4 at positions 120 (Ala/Thr) and 296 (Phe/Val) (Edelstein et al. 2014). The polymorphism at 120 is common and is distributed by race. PAR4-120A exhibited a lower reactivity and was found in 81 % of white individuals compared to 37 % of black individuals. In contrast, PAR4-120T was hyperreactive to agonists, resistant to a PAR4 antagonist, and found in 63 % of blacks compared to 19 % of whites (Edelstein et al. 2014). The frequency of Val at 296 was low and had low reactivity regardless of the amino acid at 120 suggesting that it is a dominant negative receptor. The mechanism by which the PAR4 variants elicit their distinct response to affect platelet function is not known. These polymorphisms may change the interaction of PAR4 with the membrane, allosterically alter ligand binding, or influence the transition of the receptor to an active state (Isberg et al. 2014). Structural studies examining the differences between the PAR4 sequence variants are necessary to determine the molecular basis for the differences in reactivity.

Structural Studies on PARs

The platelet field has benefited from the recent advances in membrane protein crystallography (Salon et al. 2011; Zhang et al. 2012a, 2014a, b). Included in this list is the high-resolution crystal structure of PAR1 bound to the antagonist vorapaxar (Zhang et al. 2012a). The general overall structure was similar to many other GPCRs. However, when the PAR1 structure is compared to other class A GPCRs, transmembrane helix 7 was structurally similar to activated receptors, which is surprising for an antagonist bound receptor. Complementary studies are needed to determine if this is a unique feature of PAR1 or if it is specific to the conformation induced by the antagonist vorapaxar. The experimental constraints and sequence modifications required for GPCR crystallography have thus far have prevented a detailed structural analysis of the tethered ligand mechanism (Salon et al. 2011). Recently, Alsteens and colleagues developed a modification of atomic force microscopy to probe the ligand binding site of PAR1 and determined the free-energy landscape (Alsteens et al. 2015). Based on these studies, the authors proposed a two-step binding mechanism where the ligand first interacts in a low-affinity mode and then progresses to a high-affinity mode. Further studies are necessary to determine if the two-step mechanism is shared across the PAR family and how these observations are linked to the NMR studies with the PAR1 exodomain (Seeley et al. 2003). Since PARs are grouped together by their common activation mechanism, it is tempting to use PAR1 as the preferred model to gain structural insight for other PARs. However, PARs share no more sequence identity between family members than other GPCRs in general (34–41 %). There will undoubtedly be specific information regarding activation mechanism that is unique to each PAR as more structural and biophysical data become available.

Species Differences in PARs Expression in Platelets

Animal models are widely used for preclinical studies to examine platelet function and pharmacology in vivo. There are important differences in how platelets respond to thrombin between species (Connolly et al. 1994; Derian et al. 1995). Comparative ultrastructural and functional studies of platelets show differences in the open canalicular system (OCS), cytoskeletal proteins, and regulatory proteins, which may impact the kinetics of dense granule release (Choi et al. 2010; Gruba et al. 2015). The major contributor to species-specific responses of platelets to thrombin is the repertoire of PARs expressed on their platelets.

PAR4 is expressed on the platelets of most species, whereas PAR1 and PAR3 expression is more limited. PAR1 is expressed on platelets from human, monkey, and guinea pig (Fig. 6). PAR3 is expressed on platelets from mouse, rabbit, rat, and dog (Connolly et al. 1994; Derian et al. 1995). Monkey and guinea pig are the only animal models that express both PAR1 and PAR4 on their platelets.

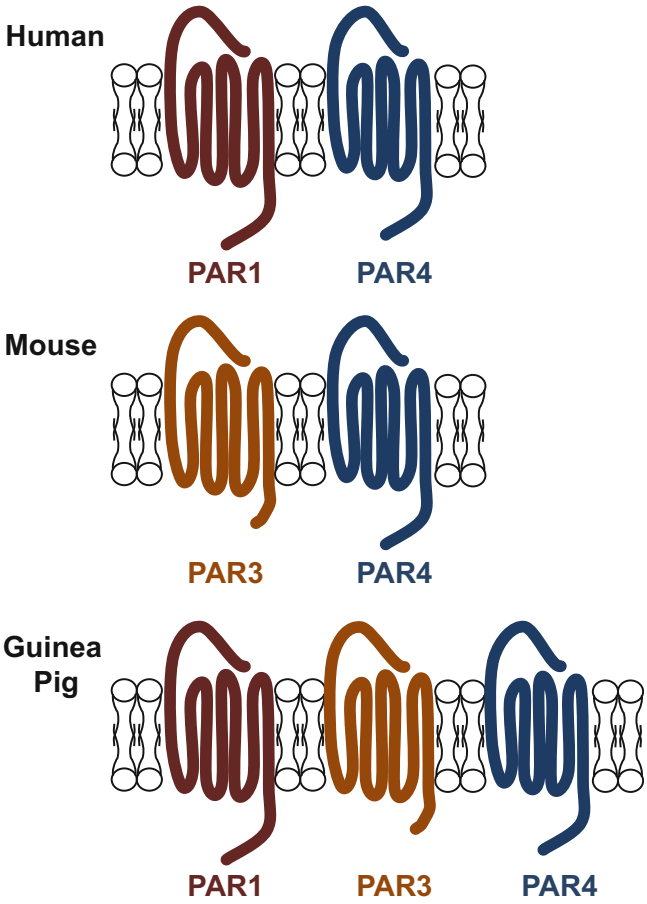

Fig. 6 Species differences in PAR expression on platelets from commonly used preclinical animal models

However, guinea pigs also express PAR3, which makes thrombin signaling more complicated in this animal model (Andrade-Gordon et al. 2001). Platelets from guinea pigs respond tenfold less to SFLLRN in aggregation experiments despite 80 % sequence identity between human PAR1 and guinea pig PAR1 (Kinlough-Rathbone et al. 1993). The high expression of PAR1, PAR3, and PAR4 influences pharmacology experiments. For example, the PAR1 inhibitor RWJ-58259 showed no significant antithrombotic effect in guinea pig thrombosis models in vivo (Andrade-Gordon et al. 2001). Taken together, guinea pigs have limitations as an animal model that need to be considered when evaluating PAR antagonists as potential antithrombotic drugs for humans. Genetically altered mice have been widely used in thrombosis studies to determine the contributions of platelet proteins in vivo. A mouse model with "humanized" PAR expression on their platelets would be an important tool for examining the specific individual contributions of PAR1 and PAR4 in vivo. Mice expressing PAR1 and PAR4 on their platelets would also serve as a convenient preclinical model for antiplatelet agents. To date, these efforts have been unsuccessful (Arachiche et al. 2014).

Take-Home Messages

- Thrombin is a potent platelet agonist that signals via proteolytic cleavage of protease activated receptors (PARs).
- PARs are G-protein-coupled receptors that signal through G_q and $G_{12/13}$ in platelets.
- PAR expression on platelets varies among species; human platelets express PAR1 and PAR4.
- PAR1 and PAR4 have distinct activation and signaling kinetics, which are influence via cooperation between PAR family members.

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The P2 Receptors

Béatrice Hechler and Christian Gachet

Abstract

Adenine nucleotides (ADP and ATP) play crucial roles in the physiological process of primary hemostasis and in the development and extension of arterial thrombosis. They act on platelets through three distinct P2 receptors: the $P2Y_1$ and $P2Y_{12}$ receptors, which are G protein-coupled ADP receptors and the $P2X_1$ receptor, a ligand-gated cation channel activated by ATP. Each of these receptors plays a distinct part in platelet activation and thrombus formation and in arterial thrombosis. Due to its central role in the amplification of platelet responses to any stimulus, the $P2Y_{12}$ receptor is a major target for currently used potent antiplatelet drugs. Platelets also express the $P2Y_{14}$ receptor which, however, does not seem to play any part in platelet physiology. Beyond hemostasis and thrombosis, platelets also strongly contribute to acute and chronic inflammatory processes including atherosclerosis, restenosis, endotoxemia, sepsis, and asthma, where these P2 receptors have distinct functions, depending on their roles in platelet function and in other blood and vascular cell types.

Introduction

Extracellular nucleotides serve as intercellular or autocrine messengers regulating numerous physiological and pathological functions including neurotransmission, muscle contraction, bone metabolism, liver glycogen metabolism, cardiac function, vascular tone, inflammation and hemostasis, and thrombosis (Burnstock and Ralevic 2014). Blood platelets were perhaps the first cells to be demonstrated to respond to extracellular nucleotides, when adenosine 5′-diphosphate (ADP) was identified 50 years ago as a small molecule released from erythrocytes which caused platelets to adhere to glass (Hellem 1960; Gaarder et al. 1961) and induced platelet aggregation (Ollgard 1961; Born 1962). The importance of ADP in platelet activation and in hemostasis and thrombosis then became rapidly recognized. Although ADP is itself a weak agonist of platelet aggregation inducing only reversible responses, it is a necessary cofactor for the normal activation of platelets by strong agonists such as thrombin, collagen, or thromboxane A_2 (TXA_2) (Cattaneo et al. 1990; Kinlough-Rathbone et al. 1992; Lau et al. 1994), which induce its release from platelet dense granules where it is stored at a very high concentration.

Stimulation of washed platelets with ADP leads to shape change, reversible aggregation at physiological concentrations of calcium, and finally desensitization (Mustard et al. 1975; Packham and Rand 2011; Gachet 2001, 2008; Gachet and Hechler 2005; Gachet et al. 2006; Hechler and Gachet 2011). Transduction of the ADP signal involves a transient rise in free cytoplasmic calcium, due to the mobilization of internal stores, a secondary store-mediated influx, and the concomitant inhibition of adenylyl cyclase activity (Gachet and Cazenave 1991). In addition, adenosine 5′-triphosphate (ATP) induces an extremely rapid influx of calcium from the extracellular medium in association with platelet shape change (Mahaut-Smith et al. 2004; Sage et al. 1997).

Prior to the molecular identification of the various platelet receptors for ADP and ATP, their intracellular signaling events were thought to be mediated either by a unique

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receptor called P2T (Hourani and Hall 1994; Gordon 1986) or by separate entities, one being coupled to the inhibition of cAMP formation (Macfarlane et al. 1983). During the early 1990s, these receptors were progressively identified. They belong to the P2 receptor family and consist of two classes of membrane receptors: P2X ligand-gated cation channels ($P2X_{1\text{-}7}$) and G protein-coupled P2Y receptors ($P2Y_{1,2,4,6,11,12,13,14}$) (Burnstock 2007). Four P2 receptor subtypes are present on blood platelets. These are the two G protein-coupled ADP receptors, $P2Y_1$ and $P2Y_{12}$, and the $P2X_1$ cation channel which is activated by ATP (Gachet 2006). Each of these receptors has a specific function in platelet activation and aggregation, which has implications for their involvement in hemostasis and thrombosis. Platelets also express the $P2Y_{14}$ receptor activated by UDP and UDP-glucose but so far its role, if any, remains elusive (Dovlatova et al. 2008) (Fig. 1).

Biology of the Platelet P2 Receptors

The $P2Y_1$ Receptor

The $P2Y_1$ receptor is broadly expressed in many cells and tissues. Its presence and role in platelets were established by the detection of mRNA in megakaryoblastic cell lines and by pharmacological studies using selective $P2Y_1$ antagonists (Hechler et al. 1998c; Léon et al. 1997). Platelets express approximately 150 $P2Y_1$ receptors per cell (Baurand et al. 2001; Ohlmann et al. 2010), which is very low as compared, for instance, to the thromboxane prostanoid (TP) receptors or the thrombin receptor PAR-1 (1000–2000 receptors/platelet) and probably explains why the signal induced by $P2Y_1$ activation is weak as compared to the signals of other G_q-coupled receptors. ADP is the preferred natural agonist of the $P2Y_1$ receptor, while ATP behaves as an antagonist in platelets

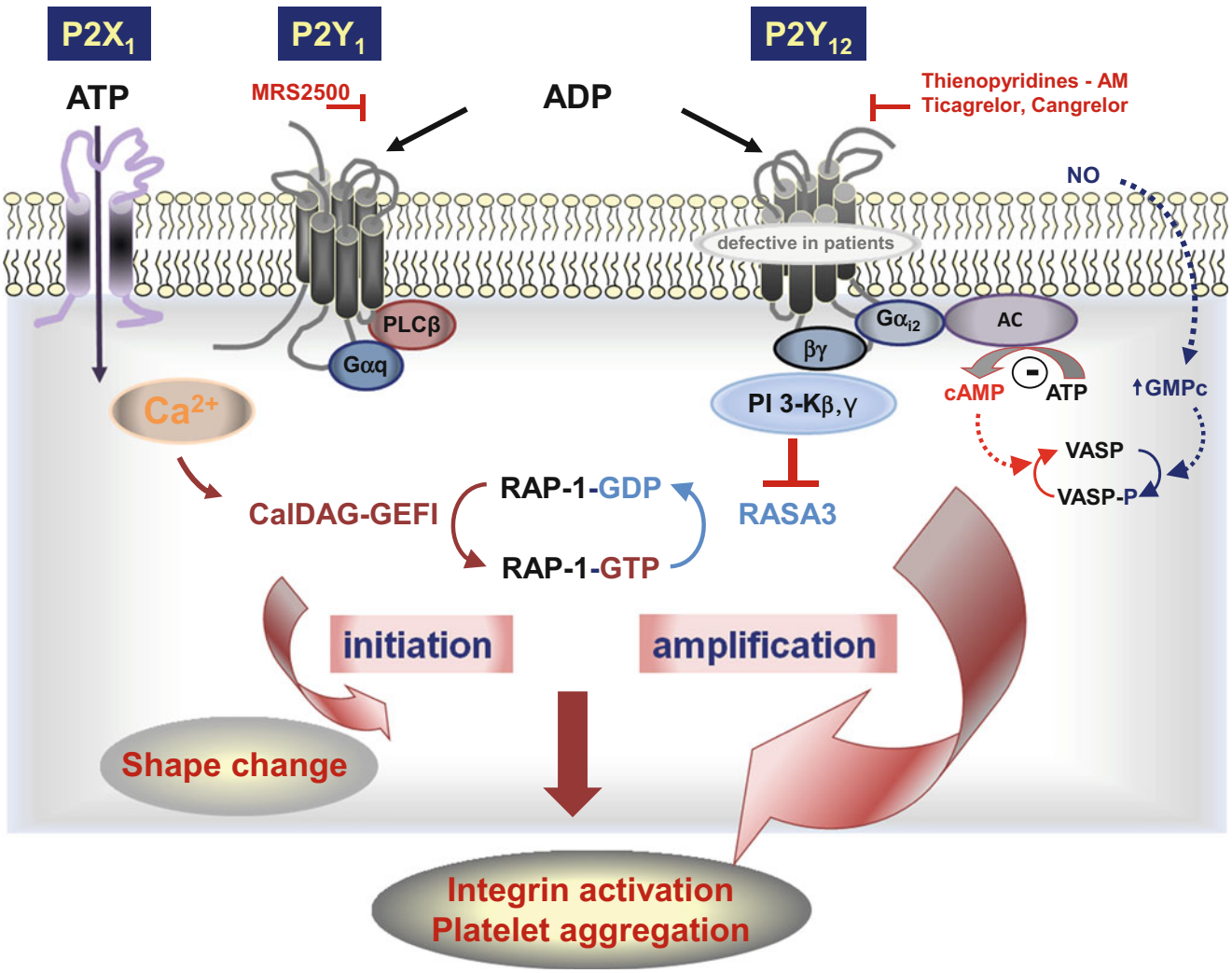

Fig. 1 Schematic illustration of the platelet P2 receptors and their respective role in platelet activation. *AM* active metabolite

(Léon et al. 1997; Hechler et al. 1998c) or as a poor partial agonist in heterologous transfected or reconstituted systems, depending on the receptor density (Waldo and Harden 2004).

The $P2Y_1$ receptor has the classical structure of a G protein-coupled receptor, and its crystal structure was reported recently (Zhang et al. 2014a, b). Its activation leads to the $G_{\alpha q}$-mediated activation of β-isoforms of phospholipase C (PLC) and to a transient increase in intracellular calcium concentrations, mainly through release of calcium into the cytoplasm from intracellular stores and additionally through influx of calcium from the external medium (Sage et al. 1990). These changes in cytosolic calcium concentration support the activation of calcium and diacylglycerol-regulated guanine-nucleotide exchange factor 1 (CalDAG-GEFI) (Crittenden et al. 2004; Stefanini and Bergmeier 2016; Stefanini et al. 2009), leading to rapid and reversible activation of the small GTPase RAP1 and integrin $\alpha_{IIb}\beta_3$ (Franke et al. 1997; Lova et al. 2002). This results in platelet shape change and weak, transient aggregation in response to ADP (Hechler et al. 1998b; Jin et al. 1998; Savi et al. 1998) (Fig. 1). Studies using $P2Y_1^{-/-}$ mice have confirmed its requirement for ADP-induced platelet shape change and aggregation (Fabre et al. 1999; Léon et al. 1999). In addition, the $P2Y_1$ receptor contributes to collagen-induced aggregation (Léon et al. 1999) and is required for collagen-induced platelet shape change when thromboxane A2 (TXA_2) formation is prevented (Mangin et al. 2004).

Platelets activated by ADP become refractory to restimulation, a phenomenon due to desensitization and internalization of the $P2Y_1$ receptor (Baurand et al. 2000, 2005; Hardy et al. 2005), through a mechanism dependent on receptor C-terminal phosphorylation sites, β-arrestin-2 interactions, and protein kinase C (PKC) activity (Hoffmann et al. 2008; Mundell et al. 2006; Reiner et al. 2009).

Overall, the $P2Y_1$ receptor mediates only weak responses to ADP but is nevertheless a crucial factor in initiating the platelet activation induced by ADP or collagen.

The $P2Y_{12}$ Receptor

The $P2Y_{12}$ receptor was cloned in 2001 (Hollopeter et al. 2001; Zhang et al. 2001). Like $P2Y_1$, it belongs to the G protein-coupled receptor family and is activated by ADP, while ATP and a wide range of its triphosphate analogues behave as antagonists (Kauffenstein et al. 2004; Bodor et al. 2003). Platelets express about 400 copies of the $P2Y_{12}$ receptor per cell (Ohlmann et al. 2013). This receptor plays a central role in the amplification of platelet responses to any stimulus, which explains why it is the molecular target of potent antiplatelet drugs including the thienopyridine compounds ticlopidine, clopidogrel, and prasugrel, which are prodrugs, and the direct antagonists ticagrelor and cangrelor (Cattaneo 2011; Michelson 2009). The $P2Y_{12}$ receptor is defective in patients with selective deficiencies of platelet activation in response to ADP (Cattaneo 2011). The tissue distribution of this receptor was long thought to be restricted to platelets and subregions of the brain. Further studies later revealed its expression and functions in microglial cells, vascular smooth muscle cells (VSMCs), dendritic cells (DCs), macrophages, and as yet unspecified leukocytes (Gachet 2012). The $P2Y_{12}$ receptor is responsible for completion of the platelet aggregation initiated by the $P2Y_1$ receptor (Hechler et al. 1998a) and for amplification of the aggregation induced by any other agents. Although ADP itself is unable to trigger secretion from platelet granules, its interaction with $P2Y_{12}$ greatly amplifies platelet secretion (Cattaneo et al. 1997, 2000) and also stabilizes platelet aggregates in vitro (Cattaneo et al. 1990; Eckly et al. 2001; Trumel et al. 1999) and thrombus formation in vivo (André et al. 2003). Conversely, $P2Y_{12}^{-/-}$ mice display a markedly prolonged bleeding time (Foster et al. 2001; Hollopeter et al. 2001), while patients with $P2Y_{12}$ deficiency may exhibit a severe bleeding diathesis (Cattaneo 2011), pointing to a central role of $P2Y_{12}$ in normal primary hemostasis (see below).

The $P2Y_{12}$ receptor activates a $G_{\alpha i2}$ G protein subtype (Ohlmann et al. 1995; Jantzen et al. 2001), which is a critical component of the signaling pathway for integrin $\alpha_{IIb}\beta_3$ activation (Jantzen et al. 2001; Hardy et al. 2004). Thus, $G_{\alpha i2}$ is responsible for the activation of two phosphoinositide 3-kinase (PI3-K) isoforms (PI3-K p110β and p110γ) which regulate $\alpha_{IIb}\beta_3$ activation (Gratacap et al. 2011; Hirsch et al. 2001; Jackson et al. 2005; Li et al. 2003; Martin et al. 2010). The downstream targets of PI3-K activation are the serine-threonine protein kinase B/Akt (PKB/Akt) and the small GTPase RAP1. This sustains $\alpha_{IIb}\beta_3$ activation (Larson et al. 2003; Lova et al. 2003, 2002; Woulfe et al. 2002) and is complementary to the CalDAG-GEFI-mediated rapid and reversible activation of RAP1 (Crittenden et al. 2004; Stefanini and Bergmeier 2016). Sustained activation of RAP1 is caused by downregulation of the RAP1 inhibitor RAP-GAP RASA3 upon stimulation of the $P2Y_{12}$ receptor (Stefanini et al. 2015). In addition, $G_{\alpha i2}$ also inhibits adenylyl cyclase activity and lowers elevated cAMP levels, which itself is not sufficient to cause platelet aggregation but facilitates it (Haslam 1973; Savi et al. 1996; Daniel et al. 1999). One may note that the inhibition of cAMP-dependent protein kinase A (PKA) leads to inhibition of the phosphorylation of the actin regulatory protein vasodilator-stimulated phosphoprotein (VASP) (Geiger et al. 1999). Although this protein does not play a key role in integrin activation, its phosphorylation state is used as a marker of the activation state of the $P2Y_{12}$ receptor, especially to monitor the effects of antiplatelet drugs inhibiting this receptor (Aleil et al. 2005; Mallouk et al. 2012) (Fig. 2).

Upon activation, a substantial fraction of membrane $P2Y_{12}$ receptors are rapidly internalized and recycled to the plasma membrane (Baurand et al. 2005). Whether this process is accompanied by a functional desensitization of $P2Y_{12}$ remains unclear. On the one hand, the $P2Y_{12}$ receptor has been shown to remain functional with conserved ability of

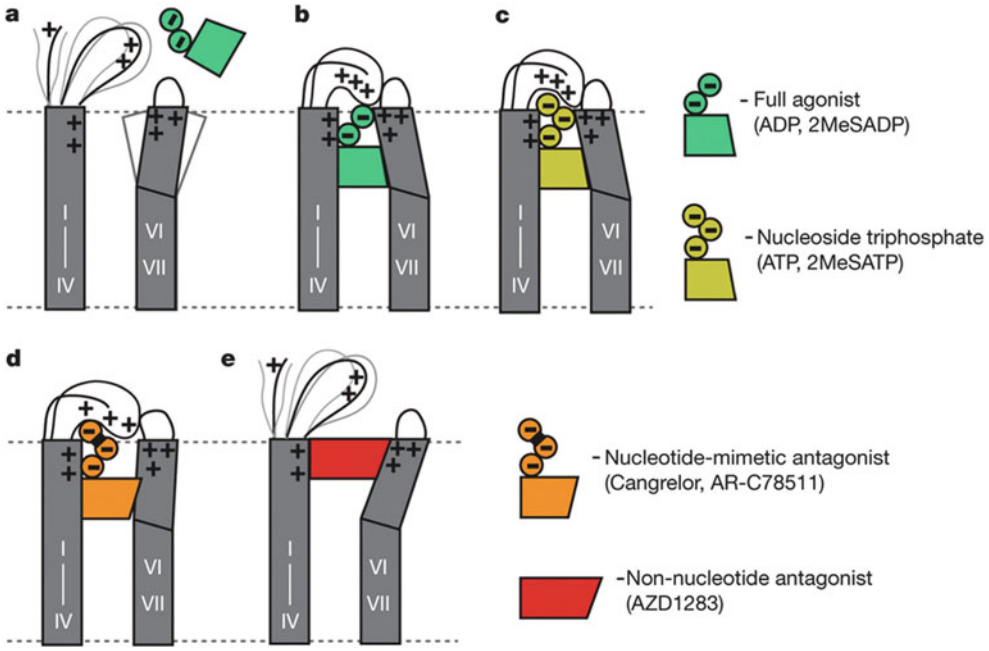

Fig. 2 Schematic illustration of conformational changes in $P2Y_{12}$ receptor extracellular region. (**a**) Unliganded (apo) state of $P2Y_{12}R$ with open entrance to the pocket and partially disordered lid. A number of partially uncompensated positive electrostatic charges among the side chains in the pocket ($R19^{Nterm}$, $K80^{2.60}$, $R93^{3.21}$, $K173^{ECL2}$, $K174^{ECL2}$, $R256^{6.55}$, and $K280^{7.35}$) disfavor formation of the stable closed state. (**b**) The closed state is stabilized by binding of nucleotide agonist (e.g., ADP, 2MeSADP). (**c**) A similar conformation with "lid" closure occurs in 2MeSATP structure and for various docked N^6 unsubstituted nucleoside triphosphate and triphosphate-mimetic ligands. (**d**) A helical reorganization is proposed for some N^6 substituted nucleoside triphosphate and triphosphate-mimetic ligands, especially with bulky N^6 substituents. (**e**) Binding of non-nucleotide antagonist AZD1283 blocks inward movement of helices VI and VII and prevents "lid" closure. Reprinted by permission from Macmillan Publishers Ltd: [Nature Publishing Group] (Zhang et al. 2014a), copyright (2014)

ADP to cause amplification of the platelet aggregation induced by other agonists (Baurand et al. 2000, 2005). The in vivo consequence is that under conditions where platelets become refractory to stimulation by ADP, the $P2Y_{12}$ receptor remains functional and is able to promote their reactivity at sites of vessel injury, thus preventing loss of their hemostatic function. On the other hand, it has been reported that $P2Y_{12}$ receptors undergo functional desensitization, through the action of G protein-coupled receptor kinases (GRK) (Hardy et al. 2005; Hoffmann et al. 2008; Mundell et al. 2006). Further studies will be required to solve the apparent contradiction raised by these reports.

Co-activation of the $P2Y_1$ and $P2Y_{12}$ receptors is necessary for a full platelet aggregation response to ADP since separate inhibition of either receptor with selective antagonists results in a dramatic decrease in aggregation (Hechler et al. 1998b; Jin et al. 1998; Savi et al. 1998). However, the $P2Y_1$ and $P2Y_{12}$ receptors are differentially involved in the platelet aggregation induced by other agonists, with $P2Y_{12}$ supporting amplification of these responses and $P2Y_1$ playing only a minor role, except in the case of collagen-induced activation. The $P2Y_1$ and $P2Y_{12}$ receptors are also differentially involved in the procoagulant activity of platelets. Both receptors are indirectly implicated through platelet P-selectin exposure and the formation of platelet-leukocyte conjugates, which leads to leukocyte-tissue factor exposure (Léon et al. 2003, 2004). Nonetheless, only the $P2Y_{12}$ receptor is directly involved in the procoagulant activity of platelets through phosphatidylserine exposure at the surface of the cells (Storey et al. 2000; Léon et al. 2001, 2003).

The $P2Y_{14}$ Receptor

Platelets also express the $P2Y_{14}$ receptor for UDP-glucose (Dovlatova et al. 2008). However, no contribution of this receptor to any platelet function has so far been observed (Dovlatova et al. 2008), and no modulation of thrombosis has been detected using $P2Y_{14}^{-/-}$ mice in various models of arterial thrombosis (unpublished data).

The Platelet $P2X_1$ Receptor

The $P2X_1$ receptor is a ligand-gated cation channel responsible for the fast calcium entry induced by ATP (Mahaut-Smith et al. 2004; Vial et al. 1997). One characteristic of this receptor is that it desensitizes very quickly, thereby hampering the in vitro study of its functions in platelet activation. Furthermore, a lack of potent and selective $P2X_1$ receptor antagonists

has hindered the search for its functional role in hemostasis and thrombosis. Nevertheless, when desensitization of the $P2X_1$ receptor is prevented by addition of high concentrations of apyrase (ATP-diphosphohydrolase EC 3.6.1.5), the selective $P2X_1$ receptor agonist alpha, beta-methylene-ATP (αβMeATP) induces a rapid calcium influx leading to a transient shape change but is unable to trigger platelet aggregation (Rolf et al. 2001) (Fig. 1). These results have been confirmed in $P2X_1^{-/-}$ mice (Hechler et al. 2003). Conversely, overexpression of the human $P2X_1$ receptor in transgenic mice did not result in aggregation or secretion in response to αβMeATP, whereas the calcium influx and shape change were enhanced (Oury et al. 2003). In addition, the $P2X_1$ receptor participates in collagen- and shear-induced aggregation (Cattaneo et al. 2002; Hechler et al. 2003; Oury et al. 2004). Interestingly, the $P2X_1$ component of the calcium signal resulting from activation of GPVI by collagen appears to be resistant to inhibitors of platelet reactivity such as endothelium-released prostacyclin or nitric oxide, which might be relevant to thrombosis when inhibitory endothelial effects remain active (Fung et al. 2012). Recent data indicate a strong contribution of neutrophils to thrombus formation under specific experimental conditions, namely, laser-induced injury of cremaster arterioles (Darbousset et al. 2014), and a key role of the $P2X_1$ receptor in this setting. Whether both platelet and neutrophil receptors are involved in this thrombotic process remains to be assessed.

Genetic Polymorphisms of the $P2Y_1$ and $P2Y_{12}$ Receptors

$P2Y_1$ and $P2Y_{12}$ have been shown to display gene sequence variations which have been proposed to be associated with variable platelet responsiveness to ADP. In $P2Y_{12}$, polymorphisms in the intronic part of the gene have been described with no obvious impact on the coding sequence. Two haplotypes have been identified, H1 and H2, the latter being proposed to be linked to enhanced platelet reactivity to ADP (Fontana et al. 2003a) and to a diminished response to clopidogrel (Staritz et al. 2009; Shalia et al. 2013) and associated with increased risks for peripheral arterial disease (Fontana et al. 2003b) and coronary artery disease (Cavallari et al. 2007). However, these results were not confirmed in latter studies (Bura et al. 2006; Lev et al. 2007; Zee et al. 2008; Ulehlova et al. 2014; Tang et al. 2013; Namazi et al. 2012; Hetherington et al. 2005; Cuisset et al. 2007). A separate single-nucleotide polymorphism of the $P2Y_{12}$ gene (34C>T; rs6785930), located in the coding region of the *P2Y12* gene, associated with an increased risk of ischemic cerebrovascular events in persons with peripheral arterial disease has been described (Ziegler et al. 2005). However, other outcomes, such as premature myocardial infarction (Motovska et al. 2010b), adverse cardiovascular events (Zee et al. 2008; Tang et al. 2013), an increased risk of bleeding (Motovska et al. 2010a), or response to clopidogrel (Ulehlova et al. 2014), were not found to be related to this single-nucleotide polymorphism. In addition, ADP-induced maximal platelet aggregation in healthy subjects was not influenced (Kim et al. 2013). It thus appears that polymorphisms of the noncoding or coding region of the *P2Y12* gene do not have a major impact if any on the receptor function or on the individual responsiveness to clopidogrel.

In $P2Y_1$, a silent polymorphism was identified at position 1622 (A/G), which led to increased platelet aggregation in response to a low concentration of ADP in healthy volunteers carrying the G allele (Hetherington et al. 2005). Again, these results were not confirmed in later studies investigating healthy volunteers (Fontana et al. 2005; Kim et al. 2013) or CAD patients treated with clopidogrel (Sibbing et al. 2006).

Congenital Defects of the Platelet P2 Receptors

Patients with congenital deficiencies of ADP-induced platelet activation experience mild to severe bleeding. To date, only $P2Y_{12}$-deficient patients have been identified, and no $P2Y_1$ or $P2X_1$ deficiency has been observed. Congenital $P2Y_{12}$ receptor defects are autosomal recessive disorders, associated with quantitative or qualitative abnormalities of the receptor. They are characterized by a marked decrease in the number of platelet-binding sites for ADP, due to mutations disrupting the expression of the receptor (Cattaneo 2011; Cattaneo et al. 1992, 2000; Fontana et al. 2009; Hollopeter et al. 2001; Nurden et al. 1995; Shiraga et al. 2005), alterations in the ligand-binding site (Daly et al. 2009), or compromised $P2Y_{12}$ receptor recycling (Nisar et al. 2011; Patel et al. 2014). Other mutations are associated with a normal number of radioligand-binding sites but a dysfunctional receptor (Cattaneo et al. 2003; Lecchi et al. 2015; Remijn et al. 2007).

Platelet P2 Receptors as Targets for Antithrombotic Drugs

Among the three platelet P2 receptors, the $P2Y_{12}$ receptor is a key target for antithrombotic drugs in clinical use, while the $P2Y_1$ and $P2X_1$ receptors are at a preclinical stage of evaluation as potential targets for new antiplatelet agents.

The $P2Y_{12}$ Receptor

The $P2Y_{12}$ receptor is one of the most important targets for antiplatelet drugs and so far the only P2 receptor subtype to be an established target for drugs in clinical use (Gachet

2012). A full chapter of this book is devoted to $P2Y_{12}$ targeting drugs, and we will only summarize some key points. As already mentioned, the $P2Y_{12}$ receptor is targeted by the thienopyridine prodrugs ticlopidine, clopidogrel, and prasugrel and the direct antagonists ticagrelor and cangrelor.

The thienopyridine compounds need to undergo hepatic metabolization to generate active metabolites, which then covalently and selectively bind cysteine residues of the $P2Y_{12}$ receptor, thereby irreversibly inhibiting the receptor and precluding the binding of ADP (Gachet et al. 1995; Mills et al. 1992; Savi et al. 1994). Large-scale clinical trials have demonstrated the beneficial effects of thienopyridines in the prevention of major cardiac events after coronary artery stent insertion and in the secondary prevention of major vascular events in patients with a history of cerebrovascular, coronary, or peripheral artery disease (PAD) (Depta and Bhatt 2015; Franchi and Angiolillo 2015). The second-generation compound clopidogrel was first compared to aspirin in the CAPRIE trial in a very large population of patients with coronary artery disease (CAD), PAD, or stroke and found to be slightly more effective in reducing the combined risk of ischemic stroke, myocardial infarction, or vascular death at a dose of 75 mg/day, designed to ensure 50 % inhibition of the platelet aggregation induced by ADP (CAPRIE-Steering-Committee 1996). The success story of clopidogrel was boosted by its combination with aspirin in a series of trials which clearly established the efficiency of this dual antiplatelet therapy, especially in the setting of percutaneous coronary intervention (PCI). However, clopidogrel was found to display weaknesses with regard to its pharmacokinetic properties, i.e., a slow onset of the effect of the drug which, despite loading doses, requires several hours to achieve optimal inhibition of platelet functions, as likewise a slow offset due to the irreversible nature of its mode of action. Moreover, the important interindividual variability in the response to this drug is still a source of intense debate concerning the monitoring or not of high platelet reactivity during treatment with $P2Y_{12}$ antagonists (Trenk et al. 2013; Cattaneo 2012). Prasugrel is a third-generation thienopyridine compound having a higher efficacy and faster onset of action than clopidogrel. This is due to a change in the molecule and a slightly different metabolic pathway, which result in a better rate of active metabolite generation as compared to clopidogrel (Cattaneo 2010). A large-scale clinical trial, TRITON-TIMI 38, including 13,609 patients planned for PCI demonstrated the overall superiority of prasugrel (60 mg loading dose, 10 mg maintenance dose) over clopidogrel (300 mg loading dose, 75 mg maintenance dose), with a 19 % reduction of ischemic events and in particular a 52 % decrease in stent thrombosis (Wiviott et al. 2007), but with a 32 % increase in major bleeding, including fatal bleeding. Although not really surprising, these results had an important impact on the practices of interventional cardiologists (Bhatt 2009). Thus, despite its limitations and before they were clearly identified, clopidogrel proved that the $P2Y_{12}$ receptor is a very attractive target for antiplatelet drugs. Consequently, extensive research has now led to multiple compounds acting in different ways. Among these, in addition to prasugrel, two new classes of molecules have emerged as alternatives to clopidogrel, namely, the oral direct antagonist ticagrelor and the intravenous short-lived compound cangrelor.

The orally active compound ticagrelor (AZD6140) is a cyclopentyltriazolopyrimidine which is a reversible antagonist of the $P2Y_{12}$ receptor and also has an inhibitory effect on the type 1 equilibrative nucleoside transporter 1 (ENT1), thereby inhibiting adenosine reuptake. The subsequent increased concentration of extracellular adenosine may have pleiotropic beneficial effects in the vasculature (Cattaneo et al. 2014). Ticagrelor (180 mg loading dose followed by 90 mg twice a day) results in profound inhibition of platelet aggregation. Despite its reversible mode of action, complete offset of the effect of the drug requires 3–5 days. Ticagrelor afforded improved cardiovascular outcomes, including a reduction in myocardial infarctions and vascular events, as compared to clopidogrel in the PLATO trial (Wallentin et al. 2009). Moreover, in this study it led to a notable decrease in cardiovascular and total mortality, which was not observed in the TRITON-TIMI 38 trial comparing prasugrel and clopidogrel. The main adverse events associated with ticagrelor are dyspnea and ventricular pauses. Both these adverse events and the beneficial effect on mortality could be related to the additional "adenosine" action of ticagrelor. Cangrelor (ARC69931MX) is an intravenously administered ATP analogue, a $P2Y_{12}$ antagonist with a very short half-life, a very rapid onset of action (3–6 min), and a very short offset (30–60 min). In the CHAMPION-PHOENIX trial comparing the standard care (aspirin plus clopidogrel) with cangrelor added to aspirin plus clopidogrel, cangrelor was clearly of benefit in the prevention of death from any cause, myocardial infarction, and stent thrombosis (Bhatt et al. 2013). Thus, cangrelor has unique properties which make it a drug for acute situations and patients waiting for surgery. It might also be helpful in patients who cannot take oral medication.

The recent resolution of the X-ray structure of the $P2Y_{12}$ receptor revealed the behavior of the receptor depending on the binding of various ligands. Especially it showed that ADP and triphosphate nucleotide antagonists induce a similar closed conformation while the non-nucleotide antagonist AZD1283 (ethyl 6-(4-[(benzylsulfonyl)carbamoyl]piperidin-1-yl)-5-cyano-2-methylnicotinate) impaired the closed conformation by blocking an inward movement of helices VI and VII (Zhang et al. 2014a, b) (Fig. 2). This compound also bound an adjacent binding pocket. These new structural data will no doubt serve as a template for the design of original new $P2Y_{12}$ antagonists acting differently as compared to the existing drugs.

The $P2Y_1$ Receptor

The generation of $P2Y_1^{-/-}$ mice has helped to reveal the key role of this receptor in arterial thrombosis, which may be surprising in view of its apparently modest role in platelet activation. Mice with $P2Y_1$ deficiency display resistance to thrombosis in various models such as systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline (Fabre et al. 1999; Léon et al. 1999) or of tissue factor (Léon et al. 2001) or localized thrombosis after ferric chloride- or laser-induced injury of mesenteric arteries (Lenain et al. 2003). Since the $P2Y_1$ receptor is also expressed by endothelial cells, mice with selective invalidation of $P2Y_1$ in either platelets or endothelial cells have been generated to elucidate the respective contributions of the $P2Y_1$ receptors of these cell types to thrombosis. Thus, mice lacking the platelet $P2Y_1$ receptor only were equally well protected in the model of ferric chloride-induced thrombosis as whole-body $P2Y_1^{-/-}$ mice (unpublished data), indicating that the platelet receptor is probably entirely responsible for the contribution of $P2Y_1$ to thrombosis.

Several selective antagonists of the $P2Y_1$ receptor have been described (Jacobson and Boeynaems 2010) which display various affinities for $P2Y_1$, namely, the adenine nucleotide analogues A2P5P, A3P5P, and A3P5PS (Boyer et al. 1996), MRS2179 (N^6-methyl-2′-deoxyadenosine 3′,5′-bisphosphate) (Baurand and Gachet 2003; Baurand et al. 2001; Boyer et al. 1998; Kim et al. 2003; Léon et al. 2001), MRS2279 (Boyer et al. 2002), and MRS2500 (2-iodo-N^6-methyl-(N)-methanocarba-2′-deoxyadenosine 3′,5′-bisphosphate) (Cattaneo et al. 2004; Kim et al. 2003). The latter displays the highest affinity for $P2Y_1$ and leads to inhibition of thrombosis in treated animals (Hechler et al. 2006). However, due to its limited bioavailability during long-term treatment, new $P2Y_1$ receptor antagonists with an improved pharmacokinetic profile need to be developed. Several novel antagonists derived from non-nucleotide chemical scaffolds have been reported, some of them being active after oral administration (Chao et al. 2013; Costanzi et al. 2012; Hu et al. 2014; Jeon et al. 2014; Morales-Ramos et al. 2008; Pfefferkorn et al. 2008; Qiao et al. 2014, 2013; Thalji et al. 2010; Wang et al. 2013; Yakovlev et al. 2014; Yang et al. 2014). Whether these compounds fulfill the criteria of stability and bioavailability in vivo will require more extensive pharmacological characterization in animal models. A function blocking antibody targeting the $P2Y_1$ receptor, called EL2Ab, has recently been described (Karim et al. 2015). EL2Ab-inhibited ADP-induced platelet aggregation in a dose-dependent manner and prevented occlusive thrombosis, in a similar way to MRS2500, when injected IV 1 h before ferric chloride injury (Karim et al. 2015).

The X-ray crystal structure of the human $P2Y_1$ receptor revealed two distinct ligand-binding sites (Zhang et al. 2015). Thus, the nucleotide antagonist MRS2500 binds to a site at the top of the TM domain between TM6 and TM7 which also involves the N terminus and extracellular loop 2. The non-nucleotide antagonist 1-(2-[2-(tert-butyl)phenoxy] pyridin-3-yl)-3-[4-(trifluoromethoxy)phenyl]urea (BPTU), instead of interacting within the TM bundle, binds to the outside of the receptor at the lipid interface between TM1, TM2, and TM3. This is the first structural characterization of a selective, high-affinity G protein-coupled receptor ligand located entirely outside the helical bundle. Clearly, this $P2Y_1$ crystal structure will open up new possibilities to target novel sites outside the conventional ligand-binding pocket for the development of new $P2Y_1$ antagonists (Fig. 3).

It is worthy of note that inhibition of the $P2Y_1$ receptor results in only moderate prolongation of the bleeding time,

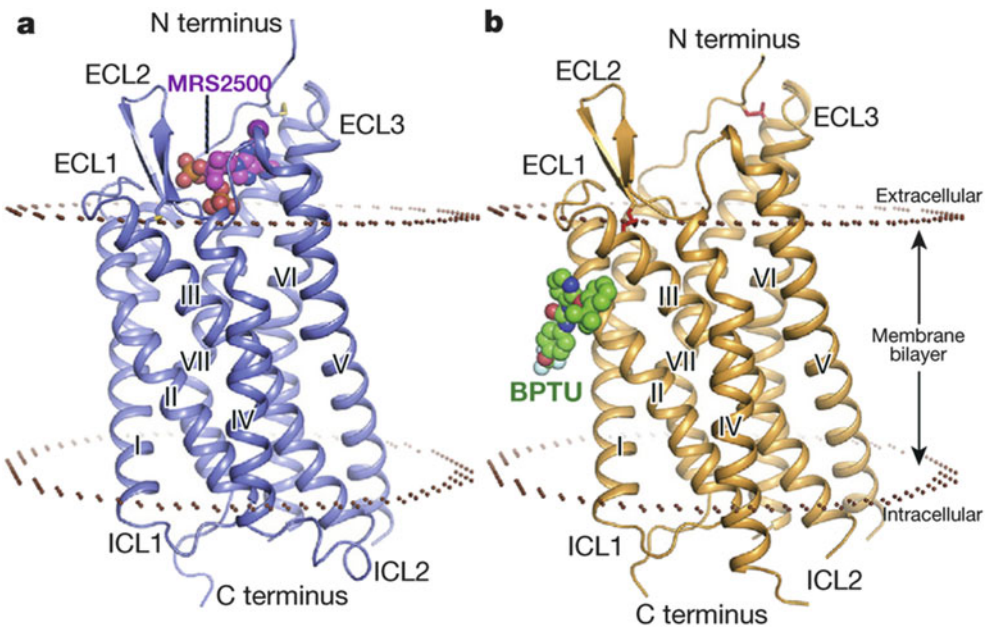

Fig. 3 Structures of the $P2Y_1R$-MRS2500 and $P2Y_1R$-BPTU complexes. (**a**, **b**) Side view of the $P2Y_1R$-MRS2500 (**a**) and $P2Y_1R$-BPTU (**b**) structures. The receptor is shown in *blue* (**a**) and *orange* (**b**) cartoon representation. The ligands MRS2500 and BPTU are shown in sphere representation with *magenta* and *green* carbons, respectively. The disulfide bonds are shown as *yellow* (**a**) and *red* (**b**) sticks. Reprinted by permission from Macmillan Publishers Ltd: [Nature Publishing Group] (Zhang et al. 2015), copyright (2015)

which could be advantageous in terms of safety as compared to inhibition of the $P2Y_{12}$ receptor. Overall, consideration of the role of $P2Y_1$ in experimental thrombosis would provide the rationale for suggesting that this receptor might be a relevant target for new antiplatelet compounds.

The $P2X_1$ Receptor

The important role of this receptor in thrombosis was not anticipated and was only revealed through in vivo evaluation of $P2X_1^{-/-}$ mice. These animals display resistance to the systemic thromboembolism induced by injection of a mixture of collagen and adrenaline and to the localized arterial thrombosis triggered by laser-induced injury of the vessel wall of mesenteric arteries (Hechler et al. 2003). $P2X_1^{-/-}$ mice are also less susceptible to the systemic thromboembolism triggered by tissue factor, i.e., in a thrombin-dependent system (unpublished data). These results were confirmed using the $P2X_1$ antagonist NF449 (Hechler et al. 2005; Kassack et al. 2004). Conversely, increased systemic thrombosis has been observed in mice overexpressing the human $P2X_1$ receptor (Oury et al. 2003). Finally, $P2X_1^{-/-}$ mice exhibit no prolongation of the bleeding time as compared to WT animals, indicating that they conserve normal hemostasis (Hechler et al. 2003). The $P2X_1$ receptor may therefore be considered as a potential target for safe antiplatelet drugs. Here also, however, the current lack of suitable selective chemical tools hampers the preclinical assessment of its relevance as compared to other targets for new antiplatelet drugs.

P2 Receptors in Inflammation

Platelets play an important part in the modulation of various inflammatory responses including chronic responses like atherosclerosis and acute responses such as sepsis, endotoxemia, or allergic asthma (Semple et al. 2011; Gawaz et al. 2005; Idzko et al. 2015). Therefore, in addition to acting as antithrombotics, antagonists and inhibitors of the various platelet P2 receptors could also have anti-inflammatory effects. Moreover, the $P2Y_1$, $P2Y_{12}$, and $P2X_1$ receptors are expressed by cells of the immune system and by vascular cells. Specifically, the $P2Y_{12}$ receptor is present on VSMCs (Harada et al. 2011; Wihlborg et al. 2004), DCs (Ben Addi et al. 2010), and some as yet poorly characterized subtypes of leukocytes (Gachet 2012); the $P2Y_1$ receptor is expressed on vascular endothelial cells, VSMCs, and leukocyte populations including macrophages (Marques-da-Silva et al. 2011), and the $P2X_1$ receptor is present on VSMCs and neutrophils (Lecut et al. 2009; Maître et al. 2015). Thus, these P2 receptors are also directly involved in the modulation of inflammation and in immune responses during thrombus formation, independently of platelet-related processes (Idzko et al. 2014; Ferrari et al. 2015).

The $P2Y_{12}$ Receptor in Inflammation

The platelet $P2Y_{12}$ receptor contributes to inflammatory responses, since reduced levels of circulating inflammatory mediators (TNFα, C-reactive protein), decreased exposure of P-selectin and CD40L, diminished formation of platelet-leukocyte aggregates, and subsequently less tissue factor exposure are observed in mice or patients receiving clopidogrel (Cattaneo 2015; Gachet 2012; Steinhubl et al. 2007). These signaling events are probably responsible for the important role of the platelet $P2Y_{12}$ receptor in restenosis (Evans et al. 2009) and atherosclerosis (Li et al. 2012; West et al. 2014). In the latter case, however, an additional contribution from the $P2Y_{12}$ receptor of VSMCs has been reported, based on studies of $P2Y_{12}^{-/-}$ mice and bone marrow chimeric mice expressing either vessel wall or platelet $P2Y_{12}$ (Li et al. 2012; West et al. 2014). In the particular form of transplant atherosclerosis due to chronic rejection of carotid artery allografts, $P2Y_{12}^{-/-}$ mice and mice treated with clopidogrel displayed reduced neointima formation in the WT grafted vessel with a reduction of infiltrating host-derived smooth muscle-like cells (SMLCs) and leukocytes (Abele et al. 2009; Harada et al. 2011). Nevertheless, in these studies it was difficult to distinguish the effects of the platelet $P2Y_{12}$ receptor from those of the receptor expressed on other cell types, namely, DCs, macrophages, or smooth muscle cells, which have been reported to display $P2Y_{12}$-dependent migration properties (Abele et al. 2009; Harada et al. 2011).

In the context of allergic asthma, platelets are required for the recruitment of inflammatory cells to the lungs and remodeling of the airway wall (Gresele et al. 1982; Idzko et al. 2015). However, divergent results have been reported concerning the role of the platelet $P2Y_{12}$ receptor. On the one hand, the $P2Y_{12}$ receptor did not appear to be involved in allergic inflammation in ovalbumin-challenged mice (Amison et al. 2015). On the other hand, in a mouse model of leukotriene E4 (LTE4)-induced asthma, the platelet $P2Y_{12}$ receptor played an important part, since the pro-asthmatic action of LTE4 was abrogated by platelet depletion or treatment with clopidogrel or in $P2Y_{12}^{-/-}$ mice (Paruchuri et al. 2009). The detailed mechanism remains to be established, although the association of $P2Y_{12}$ with an as yet unidentified coreceptor of the cysteinyl leukotriene family was invoked (Paruchuri et al. 2009). The importance of the $P2Y_{12}$ receptor in asthma is further supported by recent clinical observations. Thus, in a large family-based asthma cohort, $P2Y_{12}$ receptor variants were associated with altered lung function, while in a randomized placebo-controlled trial, prasugrel tended to decrease the

bronchial hyperreactivity to mannitol of patients with allergic asthma (Cattaneo 2015). Obviously, further investigations will be required to clarify the part played by the $P2Y_{12}$ receptor in asthma and to determine whether the discrepancies might be related to differences in the mouse models of allergic inflammation or to the use of $P2Y_{12}$ receptor antagonists versus $P2Y_{12}^{-/-}$ mice.

Recent findings point to a deleterious effect of the platelet $P2Y_{12}$ receptor in LPS-induced endotoxemia or sepsis. Indeed, clopidogrel attenuated inflammation in a rat model of LPS-induced endotoxemia (Hagiwara et al. 2011), while ticagrelor inhibited platelet-neutrophil aggregate formation, neutrophil recruitment, and lung damage in mice subjected to cecal ligation and puncture (CLP), which results in sepsis (Rahman et al. 2014). In sharp contrast, a protective role of the $P2Y_{12}$ receptor has been reported in a slightly different endotoxemia model triggered by repeated administration of LPS on four consecutive days, with $P2Y_{12}^{-/-}$ mice displaying a higher circulating neutrophil count, an enhanced inflammatory state, and more severe lung injury as compared to WT mice (Liverani et al. 2014). However, since platelet consumption was similar in WT and $P2Y_{12}^{-/-}$ mice, the authors suggested the involvement of leukocyte $P2Y_{12}$ receptors rather than the platelet receptor. Alternatively, since healthy untreated $P2Y_{12}^{-/-}$ mice displayed significantly smaller numbers of neutrophils in the spleen and bone marrow, the $P2Y_{12}$ receptor could regulate the cellular composition of the bone marrow and spleen, which might alter their responsiveness to inflammation (Liverani et al. 2014). Further studies will be needed to make all the relevant comparisons between the models, the drugs administered, and/or the use of receptor-deficient mice, in order to better characterize the role of this receptor in various settings and the impact of $P2Y_{12}$ targeting drugs.

Overall, although the data clearly demonstrate the importance of the inflammatory response in the context of arterial thrombosis, it is difficult to distinguish the contribution of the platelet $P2Y_{12}$ receptor from that of the receptor expressed on other vascular and blood cells, in the case of patients treated with any $P2Y_{12}$ targeting drug. Whether these drugs will be used beyond their current indications in atherothrombosis is not known.

The $P2Y_1$ Receptor in Inflammation

The platelet $P2Y_1$ receptor, like the $P2Y_{12}$ receptor, contributes to leukocyte activation and tissue factor exposure through its participation in platelet P-selectin exposure and the formation of platelet-leukocyte conjugates (Léon et al. 2003). These events appear to be important for the contribution of the platelet $P2Y_1$ receptor to leukocyte recruitment to the lung tissue during inflammation of the airways in allergic mice (Amison et al. 2015). However, it is likely that the $P2Y_1$ receptors present on other cell types such as leukocytes or endothelial cells also contribute to this inflammatory process. Thus, the endothelial $P2Y_1$ receptor has been found to play an important pro-inflammatory role through its contribution to the TNFα-induced upregulation of adhesion molecules (P-selectin, VCAM-1, ICAM-1), which occurs in a P38 MAPK-dependent manner and results in the recruitment of monocytes in vitro and in vivo (Zerr et al. 2011). This was confirmed in mice with selective invalidation of the endothelial $P2Y_1$ receptor (unpublished data). In addition, the endothelial $P2Y_1$ receptor contributes to the development of atherosclerosis in $ApoE^{-/-}$ mice (Hechler et al. 2008). This is somewhat paradoxical as endothelial $P2Y_1$ is known to regulate the vascular tone by activating endothelial nitric oxide synthase (Hess et al. 2009; da Silva et al. 2009), a beneficial relaxation mechanism thought to be opposed to deleterious inflammation. The blood pressure was measured in $P2Y_1^{-/-}$ mice, and no difference was found with respect to WT animals (data not shown). The signaling mechanisms underlying these effects are not fully understood but involve the transactivation of surface receptors like the EGF receptor (Buvinic et al. 2007) or tyrosine kinases such as Flt3 (Kalwa et al. 2014). Whether these mechanisms apply to the TNFα receptor remains to be investigated.

The $P2Y_1$ receptor has also been shown to be present on VSMCs, where it contributes to their proliferation and migration in vitro. This could be relevant for the intimal hyperplasia observed in a vein graft model in mice (Liu et al. 2015). In this case, a contribution from the $P2Y_1$ receptors present on platelets and macrophages cannot be excluded. Macrophages would indeed appear to be required, as their depletion abrogated hyperplasia (Liu et al. 2015), while recent data indicated a role of the $P2Y_1$ receptor in the phagocytic and migration activity of macrophages (Marques-da-Silva et al. 2011).

Altogether the $P2Y_1$ receptor could represent an attractive and original target for drugs with multiple sites of action, to treat atherothrombosis and other inflammatory diseases. Nonetheless, a potential side effect of $P2Y_1$ antagonists on vascular tone and blood pressure should be kept in mind.

The $P2X_1$ Receptor in Inflammation

As already mentioned, the $P2X_1$ receptor expressed by neutrophils contributes to thrombus formation in a context of inflammation. This has been shown in a specific model of neutrophil-dependent thrombosis of arterioles triggered by laser injury, where activation of the neutrophil $P2X_1$ receptor appeared to be indispensable for neutrophil recruitment and subsequent fibrin generation at the site of vessel injury (Darbousset et al. 2014).

One may note that the neutrophil $P2X_1$ receptor plays an important part in facilitating the neutrophil chemotaxis

induced by various chemoattractants, possibly by favoring contraction and retraction of the trailing uropod (Lecut et al. 2009). This is in accordance with its recently discovered role in endotoxemia in mice, where the $P2X_1$ receptor was found to be important for neutrophil emigration from venules and systemic inflammation, thereby contributing to tissue damage and mortality (Maître et al. 2015). Whether the platelet and/or neutrophil $P2X_1$ receptor may participate in other conditions of vascular inflammation is an open question.

Conclusion

The effects of adenine nucleotides on platelets are mediated by three distinct receptors, $P2Y_1$, $P2Y_{12}$, and $P2X_1$, each of which plays a specific role in platelet functions and arterial thrombosis. The platelet $P2Y_{12}$ receptor is an established target for antithrombotic drugs, while the $P2Y_1$ and $P2X_1$ receptors constitute attractive targets at a preclinical stage of evaluation. In addition, the platelet P2 receptor subtypes appear to be essential for the development of various pathological processes involving acute or chronic inflammation. The generation of mice presenting tissue-specific deletion of these P2 receptor subtypes will help to define their specific functions under physiological and pathological conditions. New structure-activity studies based on the crystal structures of the $P2Y_1$ and $P2Y_{12}$ receptors should help to discover more suitable antagonists. The perspective exists that these P2 receptors could also represent potential therapeutic targets for the treatment of inflammatory diseases.

Take-Home Messages

- Three distinct P2 receptors are responsible for the physiological effects of adenine nucleotides during platelet activation: the P2Y1 and P2Y12 receptors, which are G protein-coupled ADP receptors and the P2X1 receptor, a ligand-gated cation channel activated by ATP.
- The P2Y1 receptor mediates only weak responses to ADP but is nevertheless a crucial factor in initiating platelet activation by ADP or collagen.
- The P2Y12 receptor plays a central role in the amplification of platelet responses to any stimulus.
- The P2X1 receptor participates in collagen- and shear-induced aggregation.
- The P2Y12 receptor is a major target for currently used potent antiplatelet drugs.
- These three P2 receptor subtypes contribute to acute or chronic inflammation.

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Platelet Prostanoids and their receptors

Susanne Fries and Tilo Grosser

Abstract

The prostanoids, which include the prostaglandins (PGs) and thromboxane (Tx)A_2, are a family of locally acting lipid mediators. Two isozymes, cyclooxygenase (COX)-1 and COX-2, of which only COX-1 is expressed in mature platelets, form an unstable intermediate product, PGH_2, from arachidonic acid released from membrane phospholipids. At least nine PG synthases metabolize PGH_2 further to five biologically active prostanoids, which each act through one or more specific G-protein coupled receptor.

These components of the arachidonic acid biosynthetic response pathway all underlie cell-specific regulation and affect platelet biology through a diverse array of signaling events. The primary prostanoid synthesized by platelets is TxA_2, which via the TP receptor acts as an amplification signal to recruit further platelets to a growing blood clot. Patients with genetic defects in the TxA_2 pathway have a mild bleeding disorder, and pharmacological suppression of TxA_2 formation or signaling causes platelet inhibition. Suppression of TxA_2 formation is also the mechanism by which aspirin reduces the incidence of myocardial infarction and stroke. Prostacyclin (PGI_2) acting through the IP receptor is the most potent endogenous platelet inhibitor. It is formed by COX-1 and COX-2 in the vasculature and serves to constrain platelet clotting to the site of vascular injury. The inhibition of COX-2-dependent PGI_2 formation by COX-2-selective nonsteroidal anti-inflammatory drugs (NSAIDs) explains their increased risk of myocardial infarction and stroke. The role of other prostanoids in platelet biology is less established. PGD_2 is synthesized upon platelet activation in a process that involves both platelet COX-1 and plasma PGD synthase. Acting through its inhibitory DP_1 receptor on platelets, it may afford a self-regulating response to thrombogenic stimuli. PGE_2, which is formed in smaller amounts than PGD_2 during clotting, can either enhance platelet activation, via the EP_3 receptor, or inhibit platelet aggregation via the IP or possibly EP_2 or EP_4 receptors. Platelet EP receptor signaling has been hypothesized to play a role in modulating platelet function in fetal and neonatal circulation and in atherosclerosis. Prostaglandin $F_{2\alpha}$ is also formed during platelet activation by various agonists, and higher concentrations inhibit platelets through cross-reaction with other prostanoid receptors.

Prostanoid Biosynthesis

Arachidonic Acid Release The precursor of the prostanoids, arachidonic acid, is released from membrane phospholipids by the phospholipase (PL)A_2 enzymes (Burke and Dennis 2009). Group (G)IVA PLA_2 is the main isoform

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expressed in the platelet cytosol (Kramer et al. 1986). Activation by intracellular calcium and/or phosphorylation signals initiates translocation of GIVA PLA_2 to the cell membrane, where it cleaves arachidonic acid from the second carbon of the glycerol moiety of the glycerophospholipids (Channon and Leslie 1990; Lin et al. 1993; Kramer et al. 1996; Canobbio et al. 2004; Garcia et al. 2005). Vascular cells express several additional PLA_2 isoforms including calcium-dependent and calcium-independent forms, and secretory PLA_2s are found in atherosclerotic lesions (Oestvang and Johansen 2006; Alberghina 2010). Arachidonic acid released from the lipid membrane by the PLA_2s is available for prostanoid metabolism by the cyclooxygenases (COXs), which are membrane-anchored proteins (Smyth et al. 2008). Alternatively other lipid mediators in the eicosanoid class may be formed through lipoxygenase, cytochrome P450, or nonenzymatic pathways (Smyth et al. 2010), and free arachidonic acid can be reincorporated into phospholipids (Lands 2000) (Fig. 1).

Cyclooxygenases Conversion of arachidonic acid into the prostanoids PGI_2, TxA_2, PGD_2, PGE_2, and $PGF_{2\alpha}$ involves redox and rearrangement reactions by the COXs and PG synthases (Smith et al. 2011). The COXs first incorporate oxygen into arachidonic acid resulting in the formation of a labile intermediate peroxide, PGG_2, that does not leave the enzyme. In a second step, PGG_2 is reduced to the endoperoxide product PGH_2 (Smith et al. 2000). Hence, the COXs are technically PGH or PGG/H synthases. Their official gene names are prostaglandin-endoperoxide synthase 1 and 2, and their gene symbols are *PTGS1* and *PTGS2*. Catalytic function of the COXs requires dimerization, but only one monomer contains a heme group which acts as an electron source in catalyzing the formation of PGH_2 (Yuan et al. 2006). The other monomer regulates the reaction kinetics allosterically (Zou et al. 2012). COX-1 and COX-2 are highly homologous but metabolize arachidonic acid with distinct kinetics probably due to differential allosteric regulation. Thus, COX-1 is thought to be in a constantly inhibited state which requires high arachidonic acid concentrations to initiate PG biosynthesis (So et al. 1998; Smyth et al. 2005; Smith et al. 2011), while COX-2 is activated in response to much lower substrate concentrations (Chen et al. 1999). This may explain the similar contribution of both isoforms to prostanoid production in the vessel wall, although COX-1 is more abundantly expressed.

COX-2 is more readily inducible by inflammatory stimuli than COX-1 (O'Banion et al. 1992), and it is the major source of PGs mediating pain and inflammation (Smyth et al. 2005). However, COX-2 is also constitutively expressed in many uninflamed tissues (Yamagata et al. 1993; Harris et al. 1994; Breder et al. 1995; Kaufmann et al. 1996; Willingale et al. 1997). For example, both isoforms are coexpressed in uninflamed embryonic and adult blood vessels (Grosser et al. 2002; Ricciotti et al. 2013) and both contribute to vascular formation of PGI_2 (Yu et al. 2012). Mature platelets express only COX-1 (Patrignani et al. 1999). However, megakaryocytes, and immature platelets released in clinical conditions of accelerated platelet turnover, also express COX-2 (Rocca

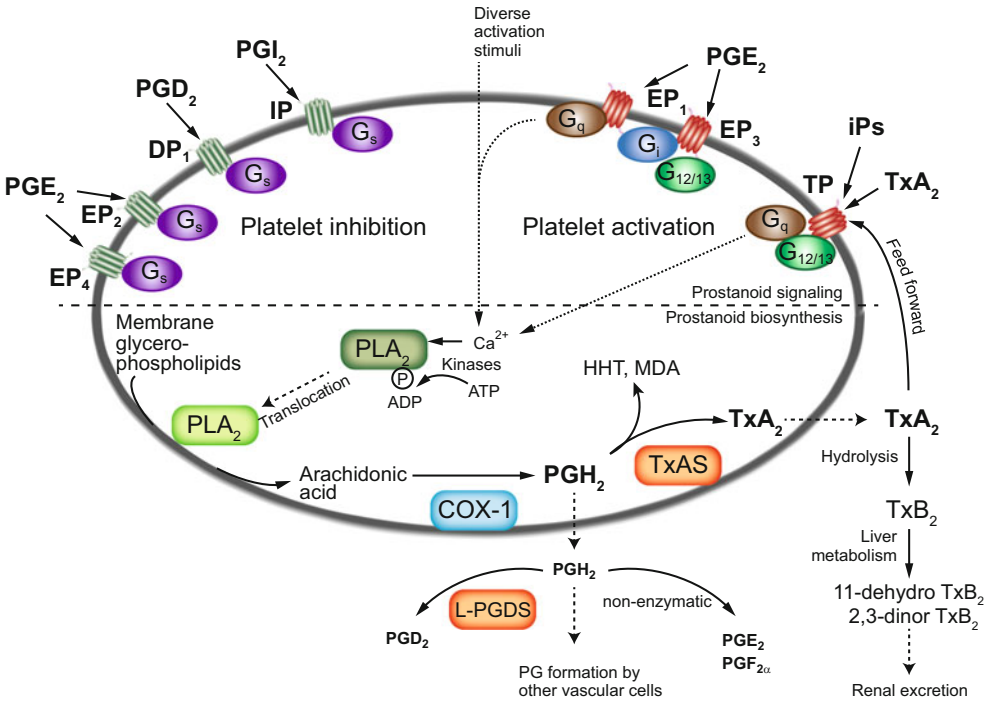

Fig. 1 Prostanoid biosynthesis and signaling in platelets. *Abbreviations*: *PG* prostaglandin, *COX* cyclooxygenase, *PL* phospholipase, *HHT* 12-L-hydroxy-5,8,10-heptadecatrienoic acid, *MDA* malondialdehyde, *iPs* isoprostanes

et al. 2002; Zimmermann et al. 2003). Splice variants of COX-2 mRNA, which would result in non- or dysfunctional proteins if translated, have been detected in mature platelets (Censarek et al. 2007). However, the role of COX-2 protein and RNA variants in platelet development and function is not understood.

Cyclooxygenase Inhibition Most NSAIDs cause substantial inhibition of platelet COX-1 (Chen et al. 2013). However, only a small number of NSAIDs, including naproxen and aspirin, cause sustained platelet inhibition. Aspirin acetylates serine-529 in the substrate-binding channel of the catalytically active COX-1 monomer (Pedersen and FitzGerald 1984). This inactivates the enzyme irreversibly and consequently depresses the formation of TxA_2 (Patrono et al. 2005; Fitzgerald and FitzGerald 2013). Because platelets are anucleate, they have only limited capacity to form new proteins. Thus, platelets exposed to aspirin are inhibited for their lifetime, and new platelets have to be released into the circulation to restore function. Inhibition of platelet COX-1 explains both aspirin's protective effect against thrombotic events and its bleeding complications (Patrono et al. 2005; Patrono 2015). COX-1 acetylation can be monitored as a direct biomarker of the pharmacological response to aspirin using mass spectrometry technology originally developed to quantify posttranslational protein modifications (Li et al. 2014; Patrignani et al. 2014). COX-1 acetylation remains close to maximal for about 2 days after a single analgesic dose (325 mg and above) of aspirin and results in complete inhibition of thromboxane formation and platelet aggregation induced by the COX-1 substrate arachidonic acid (Li et al. 2014). Cumulative inhibition of platelet Tx formation occurs over days when aspirin is administered at low doses (100 mg daily and below). This is the mechanism by which low doses of aspirin lead to platelet inhibition and consequent cardioprotection (Pedersen and FitzGerald 1984).

All other NSAIDs are reversible COX inhibitors. Naproxen is a time-dependent inhibitor with high affinity, slow dissociation kinetics, and a prolonged pharmacological effect (Rosenstock et al. 2001; Capone et al. 2005; Rimon et al. 2010; Li et al. 2014). Its half-life in the circulation is comparatively long and highly variable (ranging from 9 (Palazzini et al. 1990) to 25.7 h (Vree et al. 1993)), so that platelet inhibition following naproxen consumption may be prolonged in some patients. Piroxicam is another very long-acting, but eventually reversible COX inhibitor (Grosser et al. 2011). Naproxen and also shorter-acting NSAIDs, including ibuprofen, may compete with aspirin for access to the arachidonic acid-binding channel and prevent aspirin from acetylating its target serine residue (Catella-Lawson et al. 2001; Rosenstock et al. 2001; Capone et al. 2005; Gladding et al. 2008; Hohlfeld et al. 2008; Galliard-Grigioni and Reinhart 2009; Angiolillo et al. 2011; Anzellotti et al. 2011; Li et al. 2014). This drug-drug interaction results in a form of acquired aspirin nonresponse or "resistance" (Grosser et al. 2013).

Prostanoid Biology

Thromboxane The predominant prostanoid biosynthetic pathway in platelets is the formation of TxA_2. The membrane-bound hemoprotein thromboxane synthase (TxS) rapidly synthesizes large quantities of TxA_2 in response to platelet activation. COX-1-derived PGH_2 formation is the rate-limiting step. TxA_2 is not stored in platelets, and the biosynthetic capacity greatly exceeds the amounts needed to activate platelets (Reilly and FitzGerald 1987). This is consistent with its function as a feed-forward signal in the recruitment of additional platelets to a growing clot (FitzGerald 1991). TxA_2 is rapidly hydrolyzed ($t_{½}$ ~30 s) to the stable and biologically inactive compound TxB_2. TxB_2 is eliminated from the circulation through liver metabolism, resulting in urinary excretion of metabolites including the two most abundant compounds, 11-dehydro TxB_2 and 2,3-dinor TxB_2. A consequence of the excess Tx synthetic capacity is that the concentration-response relationship between COX-1 inhibition and functional platelet inhibition is quite steep (Reilly and FitzGerald 1987). Thus, more than 95 % of the enzymatic capacity of COX-1 must be inhibited before a change in platelet function can be expected. This explains why continuous and almost complete COX-1 inhibition, as is achieved with the irreversible inhibitor aspirin, is cardioprotective, but reversible, partial inhibition with short-acting NSAIDs such as ibuprofen is not.

Thromboxane acts through the TP receptor, which is encoded by a single gene product transcribed into two splice variants, TP_α and TP_β (Narumiya and FitzGerald 2001; Coyle et al. 2002). The TP_α protein is the predominant isoform translated in platelets, although both transcripts are detectable (Habib et al. 1999). In platelets, the TP_α interacts predominantly with G_q and $G_{12}/_{13}$. G_q activates protein kinase C-dependent pathways, which facilitate platelet aggregation, whereas G_{12}/G_{13}-mediated regulation of myosin light chain phosphorylation participates in receptor-induced platelet shape change. In expression systems, differences in G interactions and desensitization characteristics between the splice variants were observed (Vezza et al. 1999; Parent et al. 2001). Under such conditions, TP_α, but not TP_β, may couple to G_h, and TP_α may activate G_s, while TP_β interacts with G_i (Vezza et al. 1999; Parent et al. 2001). Heterodimerization of the TP with

other receptors, such as the IP, may modulate downstream signaling (Wilson et al. 2004).

Deletion of the TP receptor in the mouse prolongs bleeding time, renders platelets unresponsive to TP agonists, and modifies their response to collagen, but not to ADP (Thomas et al. 1998). Similar phenotypes were described in humans with genetically disrupted TP signaling. For example, a mutation in the first intracellular loop of the TP receptor is associated with a mild bleeding diathesis and resistance of platelet aggregation in response to TP agonists (Hirata et al. 1994). The TP undergoes rapid desensitization following its own activation (homologous desensitization), or through other mediators (heterologous desensitization), which terminates the receptor response (Nakahata 2008). This involves TP phosphorylation followed by vesicular internalization and subsequent downregulation of the receptor protein (Nakahata 2008). In addition to TxA_2, isoprostanes (iPs), which are free radical-catalyzed nonenzymatically formed products of arachidonic acid, may activate the TP (Audoly et al. 2000). Prevention of TP activation through iPs despite suppression of TxA_2 by aspirin was the rationale underlying the development of long-acting TP receptor antagonists as platelet inhibitors (Fig. 2). However, early attempts to develop such molecules failed. Persistent and complete platelet inhibition (as is afforded by irreversible acetylation of COX-1 by aspirin) was not achieved due to inadequate pharmacodynamic properties. More recently, the TP receptor antagonist terutroban was found to be similarly effective, but not superior to aspirin in the prevention of stroke (Bousser et al. 2011). Its development was halted in view of the equal efficacy and lower cost of aspirin.

TxS also converts PGH_2 to 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA) (Diczfalusy et al. 1977). HHT activates the leukotriene B_4 receptor type 2 (*BLT2*) and is thought to play a role in wound healing (Liu et al. 2014). MDA, which is produced in approximately equimolar amounts with TxA_2, forms protein adducts that can cause peptide cross-links (Zagol-Ikapite et al. 2015). However, whether this affects platelet function is not known.

Prostaglandin D_2 While PGH_2 is an unstable endoperoxide with a half-life of about 5 min in aqueous solutions (Svensson et al. 1975) and less in plasma where it is bound by proteins (Maclouf et al. 1980), it can act as a substrate for PG synthases expressed in plasma or other cells, a process sometimes referred to as endoperoxide transfer or shift (Mayeux et al. 1989; Boilard et al. 2011). PGD_2 formation during platelet activation relies on such a mechanism. There are two distinct PGD synthases, hematopoietic PGDS (H-PGDS), which is found in mast and other immune cells, and the lipocalin-type PGDS (L-PGDS), which is localized in many tissues and in plasma (Song et al. 2012). Platelets do not express the PGD synthases, but L-PGDS in plasma forms PGD_2 using PGH_2 released during platelet activation (Oelz et al. 1977; Song et al. 2012). PGD_2 signals through two receptors, DP_1 and DP_2, of which DP_1 is expressed in human, but not mouse platelets. DP_1 couples primarily to G_s resulting in adenylate cyclase stimulation and platelet inhibition. DP_2 couples to G_i. The DP_1 expressed in platelets, like the IP (see below), may function as a limiting homeostatic response to thrombogenic stimuli. Thus, combining a DP_1 receptor antagonist, laropiprant (Fig. 2), with niacin to limit the contribution of PGD_2 to the flushing response induced by niacin may have undermined the putative beneficial effect of niacin on cardiovascular risk by increasing platelet activity in the HPS2-THRIVE trial (Song et al. 2012; Song and FitzGerald 2013). While it is unclear if this was the cause of the failure of the HPS2-THRIVE trial to show effectiveness of the niacin/laropiprant combination (Group et al. 2014), the combination product has been withdrawn from the market in countries where it was approved.

Prostacyclin Prostacyclin (PGI_2) is continuously released into the circulation at a relatively constant rate and quickly ($t_{½} < 3$ min) degraded hydrolytically to 6-keto $PGF_{1\alpha}$ (FitzGerald et al. 1981). Like TxB_2, 6-keto $PGF_{1\alpha}$ is subject to liver metabolism, and a major urinary metabolite, 2,3-dinor-6-keto-$PGF_{1\alpha}$, allows monitoring of PGI_2 formation in the organism (Brash et al. 1983). The hemoprotein PGI_2 synthase (PGIS), which catalyzes the isomeric rearrangement of PGH_2 to PGI_2, was originally purified from bovine aorta (DeWitt and Smith 1983) and is constitutively expressed in endothelial and vascular smooth muscle cells (Wu and Liou 2005). COX-1 and COX-2 are also constitutively expressed in the vasculature, and both contribute to the formation of PGI_2 (Ricciotti et al. 2013). Highly controlled clinical studies with COX-2 inhibitors in healthy volunteers showed that 40–60% of the whole body PGI_2 production is COX-2 dependent (Catella-Lawson et al. 1999; McAdam et al. 1999; Fries et al. 2006). Experiments in mice, in which COX-2 was selectively deleted in endothelial or vascular smooth cells or both, identified the vasculature as the predominant source of COX-2-derived PGI_2 formation (Yu et al. 2012). In situations of accelerated platelet-vessel wall interactions such as unstable angina and myocardial infarction, PGI_2 formation is highly upregulated and coincides with platelet TxA_2 release (FitzGerald et al. 1984, 1987). Endoperoxide transfer from platelets to vascular cells may contribute at least partially to this increase in PGI_2 formation. The PGI_2 response is thought to act as a localized negative feedback signal that constrains the thrombus to the site of endothelial injury (Cheng et al. 2002), because PGI_2 inhibits platelet activation through its specific receptor, the IP. The IP is coupled to

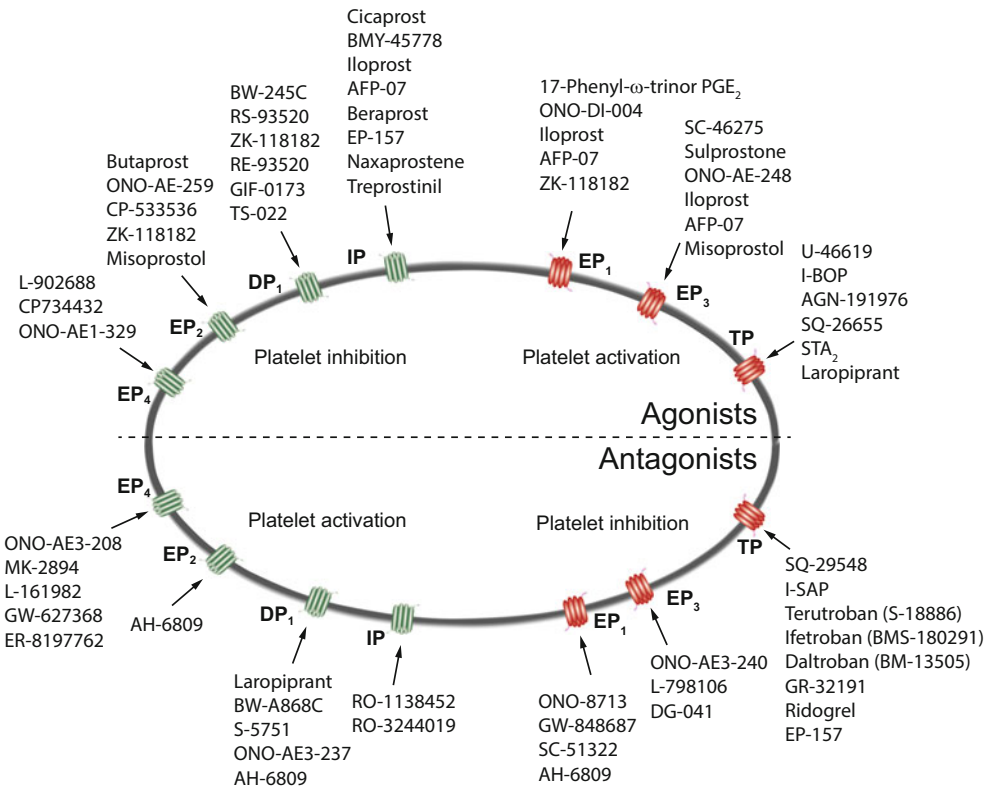

Fig. 2 Prostanoid receptor agonists and antagonists. Prototypic prostanoid receptor ligands and their primary target are listed. Many compounds have limited specificity. Molecules with pronounced effects on multiple prostanoid receptors are shown with multiple receptors. The specificity and potency of prostanoid receptor agonists and antagonists have been reviewed in more detail by Woodward et al. (2011)

G_s-adenylyl cyclase. The resulting cAMP increase activates protein kinase A (PKA), which, among a number of target proteins, deactivates myosin light chain kinase leading to reduced myosin phosphorylation and decreased platelet aggregation. PKA also phosphorylates the vasodilator-stimulated protein (VASP), which regulates fibrinogen binding by modulating the inside-out signaling of the integrin $\alpha_{IIb}\beta_3$, the major fibrinogen receptor in platelets. PGI_2 may also affect the hemostatic system by regulating the expression of thrombin receptors and thrombomodulin (Schror et al. 2010).

Disrupting PGI_2 signaling in mice by deleting its specific receptor, the IP receptor, predisposes to thrombosis in vivo (Murata et al. 1997; Cheng et al. 2002), and various models have been used to demonstrate that perturbation of PGI_2 function augments the thrombotic response. Thus, IP deletion accelerated the thrombotic occlusion of large arteries in response to injury in a gene-dose-dependent fashion (Cheng et al. 2006). Administration of a selective COX-2 inhibitor at doses that reduced PGI_2 formation also resulted in a shortened vascular occlusion time (Cheng et al. 2006). Similarly, genetic disruption of COX-2 or partial genetic disruption, which would be expected to mimic pharmacological inhibition more faithfully than complete knockout, reduced PGI_2 formation and accelerated thrombosis (Cheng et al. 2006; Cryan et al. 2006; Cathcart et al. 2008; Seta et al. 2009). COX-2-derived PGI_2 formation was mapped to the vasculature using tissue-specific deletion of COX-2 in endothelial cells and vascular smooth muscle cells (Yu et al. 2009). Indeed, tissue-specific deletion of COX-2 in vessel wall cells was sufficient to reduce PGI_2 biosynthesis and augmented the thrombotic response in a microvascular laser injury model (Yu et al. 2009). Finally, selective inhibition of COX-2 depressed PGI_2 biosynthesis and predisposed rats and hamsters to platelet activation and arterial thrombosis (Buerkle et al. 2004; Pidgeon et al. 2004). All these observations are consistent with findings from human studies. Thus, polymorphisms in COX-2, PGI_2 synthase, and IP receptor have variously been associated with adverse cardiovascular outcome after angioplasty and hypertension (Arehart et al. 2008; Kohsaka et al. 2008; Lemaitre et al. 2009) and the inhibition of COX-2-dependent PGI_2 formation by COX-2-selective NSAIDs is associated with an increased risk of myocardial infarction and stroke (Grosser et al. 2006, 2010).

Other Prostanoids PGH_2 can spontaneously rearrange to form other prostanoids, and this nonenzymatic process might be the major source of the comparatively small amounts of PGE_2 and $PGF_{2\alpha}$ generated during platelet activation; the E and F synthases are not expressed in platelets nor are they known to be present in plasma in significant amounts. $PGF_{2\alpha}$ may inhibit platelets through cross-reaction with other prostanoid receptors (Armstrong et al. 1985). PGH_2 itself can act as an agonist on the TxA_2 receptor. The role of PGE_2 in platelet function in vivo remains less

clear. In vitro, high PGE_2 concentrations (>10 μM), acting via the IP or, theoretically, the EP_2 or EP_4 receptor, inhibit platelet function (Fabre et al. 2001). Low concentrations of PGE_2, acting via the EP_3, augment submaximal platelet stimulation by other agonists (Andersen et al. 1980; Fabre et al. 2001; Schlagenhauf et al. 2015). Consistent with the latter observation, mice lacking the EP_3 receptor have an increased bleeding tendency and decreased susceptibility to thromboembolism (Ma et al. 2001). Deletion of the enzyme primarily mediating inflammatory PGE_2 formation, the mPGES-1, did not affect thrombogenesis in vivo, probably due to substrate rediversion and augmented formation of PGI_2 (Cheng et al. 2006). Platelet EP receptor signaling has been hypothesized to play a role in modulating platelet function in fetal and neonatal circulation and in atherosclerosis (Tilly et al. 2014; Schlagenhauf et al. 2015).

Take-Home Messages

The primary prostanoid synthesized by platelets is thromboxane (Tx) A, which via the TP receptor acts as an amplification signal to recruit further platelets to a growing blood clot. TP antagonists have been clinically developed as platelet inhibitors but were abandoned in view of the proven efficacy and lower cost of aspirin.

Prostacyclin (PGI) acting through the IP receptor is the most potent endogenous platelet inhibitor. Inhibition of vascular PGI contributes to the prothrombotic adverse effects of cyclooxygenase-2 inhibition by non-steroidal anti-inflammatory drugs.

The role of other prostanoids in platelet biology is less established.

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Platelet Genomics

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Abstract

Human platelets are anucleate cells that contain no genomic DNA although they contain both mRNAs and microRNAs derived from precursor megakaryocytes. Nonetheless, genetic variability encompassed within both intronic and exonic sequences are clearly implicated in a wide range of functional parameters known to regulate gene expression and protein function, with concomitant effects on platelet reactivity, volume, peripheral counts, and cellular responsiveness (Hematol Oncol Clin North Am 27:443–463, 2013; Semin Thromb Hemost 39:291–305, 2013; Curr Opin Hematol 19:371–379, 2012). In this chapter, we will review the current status of genetic variations that have been implicated in functional platelet responsiveness, many of which have recently been identified using genome-wide association studies (GWAS) (Genes 5:51–64, 2014; Nat Rev Genet 11:241–246, 2010; Curr Opin Genet Dev 23:339–344, 2013). For synergistic information relevant to platelet genetics, the reader should refer to accompanying chapters on platelet mRNAs (Rowley et al. 2017), platelet noncoding RNAs (Edelstein and Bray 2017), and transcriptomic changes associated with thrombotic risk (Gnatenko et al. 2017).

Many genetic variants have subtle effects and it is likely that complex platelet-related phenotypes associated with cerebro- or cardiovascular risk result from additive effects from multiple loci, contributing to a final common pathway for defective platelet functional and clinical outcome (J Thromb Haemost 5:188–195, 2007; Curr Vasc Pharmacol 9:479–489, 2011; J Thromb Thrombolysis 32:201–208, 2011). Thus, a single subtle effect may be difficult to delineate or consistently reproduce across cohorts. In addition, other confounding variables (e.g., age, sex, body mass index, alcohol consumption, concomitant drugs, nutritional status, and environmental pollutants) may also affect platelet function (Thromb Haemost 105:S60–S66, 2011). Thus, well-defined phenotypes provide the most informative outcomes. Additionally, GWAS with large cohort sizes provide the most robust information relevant for reliable identification of new genetic variations with subtle effect (s) on platelet functional parameters (Fig. 1).

Inherited Genetic Disorders Associated with Platelet Functional Variation

Congenital platelet disorders usually present with mild to severe bleeding typically associated with quantitative changes in platelet counts. While the diagnosis of severe disorders is relatively clear, subtle platelet dysfunction may be much more difficult to interpret, and congenital

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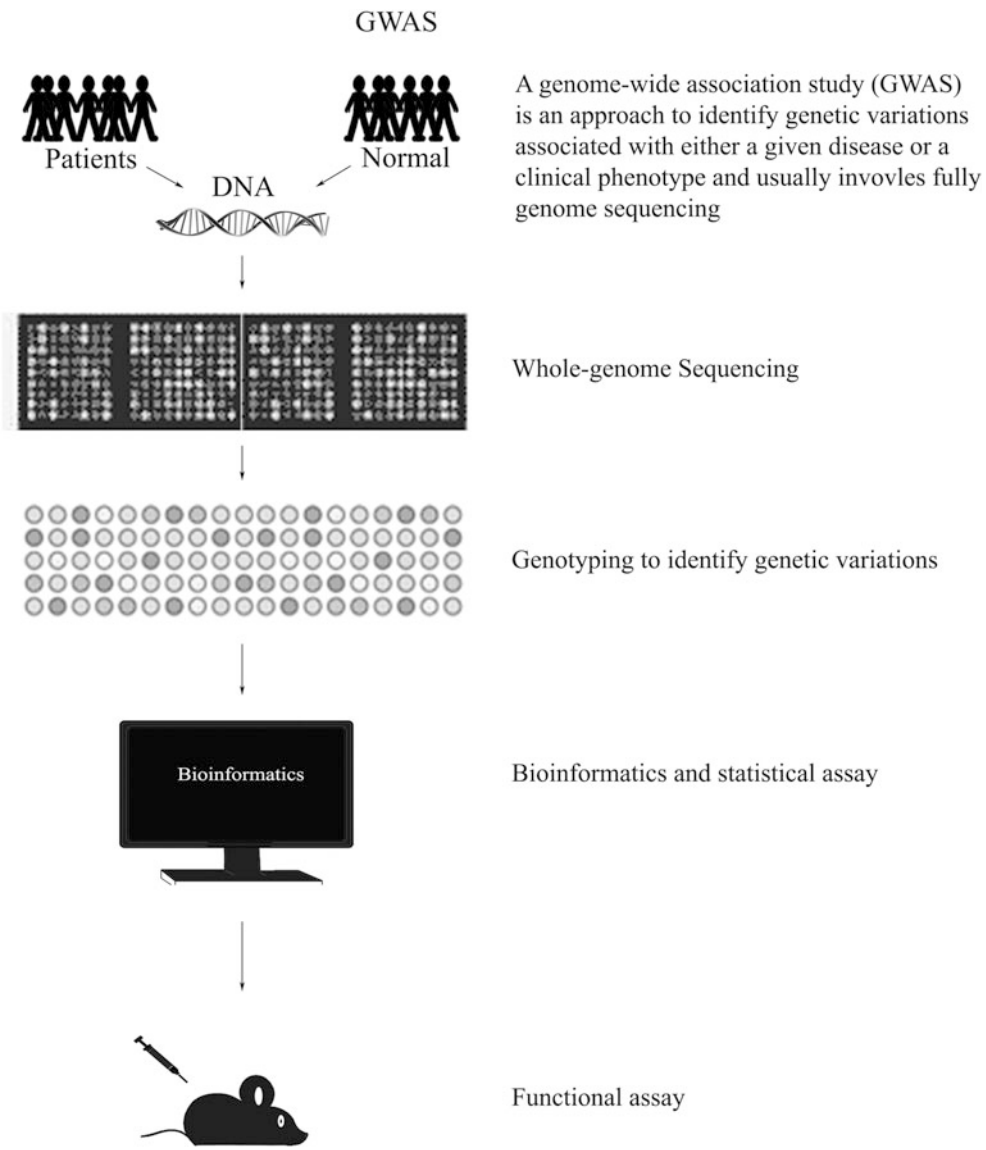

Fig. 1 Genome-wide association studies (GWAS). DNA from patients and normal controls will be used for whole genome sequencing analysis. Single-nucleotide polymorphisms (SNPs) as well as other genetic variations will be compared between two groups in bioinformatics correlation study. Candidate genes will be then tested in functional study

thrombocytopenia may be misclassified as immune thrombocytopenia or ignored because of mild changes in the platelet count. In this section, we provide an update on newly identified genes involved in inherited platelet defects (Table 1). The reader is also referred to well-characterized genetic defects that have been reviewed comprehensively in previous publications (Lambert 2015; Nurden et al. 2012; Israels and Rand 2013; Songdej and Rao 2015). Genes involved in inherited platelet disorders are associated with defects in cell signaling, transcriptional regulation, granule biogenesis, and the cytoskeleton; many patients with these disorders also present with systemic symptoms other than platelet dysfunction.

Cytoskeleton Genetic Disorders

MYH9. *MYH9*-related disorders are an autosomal dominant form of macrothrombocytopenia with giant platelets and platelet counts range from 10 to 136 ($\times 10^9$ L^{-1}) (Kunishima and Saito 2010). In patients with *MYH9*-related disease, more bleeding occurred in patients harboring mutations in the motor domain versus the tail domain of *MYH9*, and 40 % of patients had at least one extra-hematological manifestation such as renal disease, hearing loss, or cataracts (Saposnik et al. 2014).

FLNA. Actin binding protein 280 is coded by *FLNA* on the X-chromosome (Falet 2013). It tethers GP1bα and integrin $\alpha_{IIb}\beta_3$ to the underlying cytoskeleton in platelet,

Table 1 Inherited platelet disorders

Mechanism	Gene	Disorder	Inheritance	Platelet count	Platelet size	Platelet function
Cytoskeleton	MYH9	MYH9 RD	AD	Low	Large	Abnormal
	FLNA	Filaminopathies	X-linked	Low	Large	Mildly affected
	TUBB1	TUBB1 RD	AD	Low	Large	Normal
	ACTN1	ACTN1 RD	AD	Low	Large	Normal
Transcription	FLI1	Paris–Trousseau/Jacobsen	AD	Low, recovers around first year	Large	Mildly affected
	RUNX1	FPD/AML	AD	Moderately low	Normal	Mildly affected
	ETV6	Thrombocytopenia with predisposition to hematologic malignancy	AD	Variable (elevated MCV and predisposition to leukemia)	Normal	Mild to moderate bleeding
	GFI1B		AR	Low (also may have red cell abnormalities)	Large	Moderately affected
Granule	NBEAL2	Gray platelet syndrome	AR	Low	Large	Affected
Signaling	PRKACG		AR	Low	Large	Abnormal
	RBM8A	TAR syndrome	AR	Very low but improves over time	Normal	May be mildly affected
	5′UTR ANKRD26 or ANKRD26	ANKRD26-RD	AD	Moderately low	Normal	Mildly affected/ normal

thereby resulting in disruption of the platelet plasma membrane under high shear. Defects involving *FLNA* have been associated with bleeding and low platelet counts in patient subsets. Recently, mutations of *FLNA* have been associated with an isolated platelet phenotype and the role of *FLNA* in platelet and megakaryocyte has been explored further (Nurden et al. 2011; Begonja and Pluthero 2015).

TUBB1. Tubulin B1 (*TUBB1*) is involved in an autosomal dominant macrothrombocytopenia (Balduini and Savoia 2012; Pecci 2013). A polymorphism in *TUBB1* (rs121918555) has been associated with lack of response to ITP therapy (Kunishima et al. 2014; Basciano et al. 2013). A genome-wide association study of more than 65,000 individuals of European ancestry demonstrated that the *TUBB1* locus was a major determinant of mean platelet volume (Gieger et al. 2011).

ACTN1. Actin-1 (*ACTN1*) mutations were described in six Japanese pedigrees with congenital macrothrombocytopenia after whole exome sequencing (WES) (Kunishima et al. 2013). These families all had autosomal dominant macrothrombocytopenia with platelet counts ranging from 54 to 132 ($\times 10^9$ L^{-1}) and a mean increase in platelet size of 30 % with platelet anisocytosis; increased expression of GPIβ/IX and $\alpha_{IIb}\beta_3$ was thought to be due to increased platelet size. A recent study using WES added more families with *ACTN1* mutations in a large cohort of patients with thrombocytopenia (Westbury et al. 2015).

Transcription Genetic Disorders

FLI1. Paris–Trousseau (Jacobsen) syndrome is an autosomal dominant macrothrombocytopenia (Songdej and Rao 2015; Tijssen and Ghevaert 2013). It is believed that this disease is due to deletion of a portion of the distal portion of long arm of chromosome 11 (11q23.3-24) where the transcription factor Friend of Leukemia Integration (*FLI1*) locus is found. Patients with Paris–Trousseau (Jacobsen) syndrome have giant α granules in a proportion of their platelets (1–5 %) and have a mild bleeding phenotype with mild platelet aggregation defects. In addition, patients with larger deletions of 11q23.3-24 have mental retardation, trigonocephaly, dysmorphic facies, congenital heart disease, and abnormalities of the urogenital and skeletal system (Mattina et al. 2009).

RUNX1. Mutations in Runt-related transcription factor-1 (*RUNX1*) lead to a familial platelet disorder with predisposition to acute myeloid leukemia, and an increased incidence of thrombocytopenia with normal platelet size and platelet dysfunction with abnormal aggregation responses and abnormal secretion (Songdej and Rao 2015; Tijssen and Ghevaert 2013; Owen et al. 2008). Most patients present with mucocutaneous bleeding symptoms: easy bruising, epistaxis, and bleeding after minor surgery or dental challenges, but the penetrance is variable and unaffected family members carrying mutations in involved families are reported (Stockley et al. 2013).

ETV6. Mutations of Ets variant 6 (*ETV6*) have been associated with autosomal dominant thrombocytopenia of varying degree, erythrocyte macrocytosis, and various hematologic malignancies including B-cell acute lymphocytic leukemia, multiple myeloma, chronic myelomonocytic leukemia, T-cell/myeloid mixed phenotype leukemia, refractory anemia with excess blasts, and myelodysplastic syndrome (Zhang et al. 2015; Songdej and Rao 2015; Tijssen and Ghevaert 2013). In addition, several family members were also reported to have skin cancer and colorectal cancer (Noetzli et al. 2015).

GFI1B. Growth factor independent 1B (*GFI1B*) is a transcription factor previously thought to be important in murine erythroid and platelet development (Songdej and Rao 2015; Tijssen and Ghevaert 2013). Mutations in *GFI1B* lead to mild clinical bleeding, red blood cell anisopoikilocytosis, and macrothrombocytopenia, with absent α granules and abnormal aggregation responses (Stevenson et al. 2013).

Granule Biogenesis Genetic Disorder

NBEAL2. Mutations involving myelofibrosis Neurobeachin-like 2 (*NBEAL2*) have been causally identified as the molecular basis for the gray platelet syndrome (GPS) (Freson et al. 2014). *NBEAL2* deficiency results in defective α-granule biogenesis and impaired inflammatory response in a murine model (Deppermann et al. 2013). Granule biogenesis-associated platelet defect usually is a part of a larger syndromic disease such as Hermansky–Pudlak syndrome (mutations in *HPS* genes), arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome with platelet dysfunction. Many patients with gray platelet syndrome develop stable myelofibrosis (Albers et al. 2011; Kahr et al. 2011; Gunay-Aygun et al. 2011).

Cell Signaling Genetic Disorders

PRKACG. The *PRKACG* gene encodes the γ subunit of cyclic-adenosine monophosphate (c-AMP)-dependent kinase A (PKA) and it is associated with autosomal recessive thrombocytopenia with defects in proplatelet formation and platelet activation (Landolt-Marticorena and Kahr 2014; Nurden and Nurden 2015). These patients have severe macrothrombocytopenia ($5–8 \times 10^9$ L^{-1}) and significant bleeding (Manchev et al. 2014). Genetic sequencing revealed the earlier mutation and biochemical studies confirmed that family members who were homozygous for the mutation had decreased *FLNA* in megakaryocytes and platelets, and increased levels of platelet cAMP; patients who were heterozygous had intermediate levels compared to controls.

RBM8A. Thrombocytopenia absent radii (*TAR*) syndrome is different from congenital amegakaryocytic thrombocytopenia (CAMT). TAR syndrome presents at birth with thrombocytopenia, which typically is associated with skeletal abnormalities and improvement in the platelet count sometime in the first 5 years of life (Klopocki et al. 2007; Fiedler et al. 2012). Proximal microdeletions in 1q21.1 and low frequency noncoding SNPs in the 5′UTR of *RBM8A* (RNA Binding Motif Protein 8A) were identified to be associated with TAR syndrome (Albers et al. 2013). TAR syndrome seems to be due to reduced expression of Y14 (the protein encoded by *RBM8A*) (Albers et al. 2012). Patients with TAR syndrome have very high thrombopoietin levels and impaired phosphorylation of Jak2 kinase that appears to improve with age corresponding to improved platelet counts.

ANKRD26. Recent report shows that the mechanism of thrombocytopenia associated with mutations in the 5′UTR of Ankyrin repeat domain 26 (*ANKRD26*) is due to the loss of appropriate *ANKRD26* silencing and persistent TPO/MPL pathway signaling (Bluteau et al. 2014). *RUNX1* and *FLI1* bind to the 5′UTR region of *ANKRD26* to inhibit the expression of *ANKRD26* in late megakaryocytes. With mutations in 5′UTR of *ANKRD26*, RUNX1 and FLI1 are unable to bind and the expression of *ANDRD26* remains high, resulting a defect in proplatelet formation due to increased signaling through the TPO/MPL pathway (Bluteau et al. 2014).

Platelet Receptor Genetic Polymorphism Affecting Functional Responses

In general, polymorphism involving the three major platelet receptors (ADP receptors, collagen receptors, and fibrinogen receptors) has minimal disease-relevant effects on platelet function (Floyd and Ferro 2012; Williams et al. 2010). However, the correlation between these receptors polymorphism with antiplatelet drug response has also been studied and will be discussed further in the GWAS antiplatelet drug response section (*vide infra*) (Quinn and Topol 2001).

ADP Receptors

P2Y1 and P2Y12 are the two major adenosine diphosphate (ADP) receptors (Fig. 2). Individual variability of ADP-induced platelet aggregation and fibrinogen binding in healthy volunteers and patients has been previously demonstrated. Five different genetic polymorphisms for the P2Y12 gene and two haplotypes H1 and H2 with frequencies

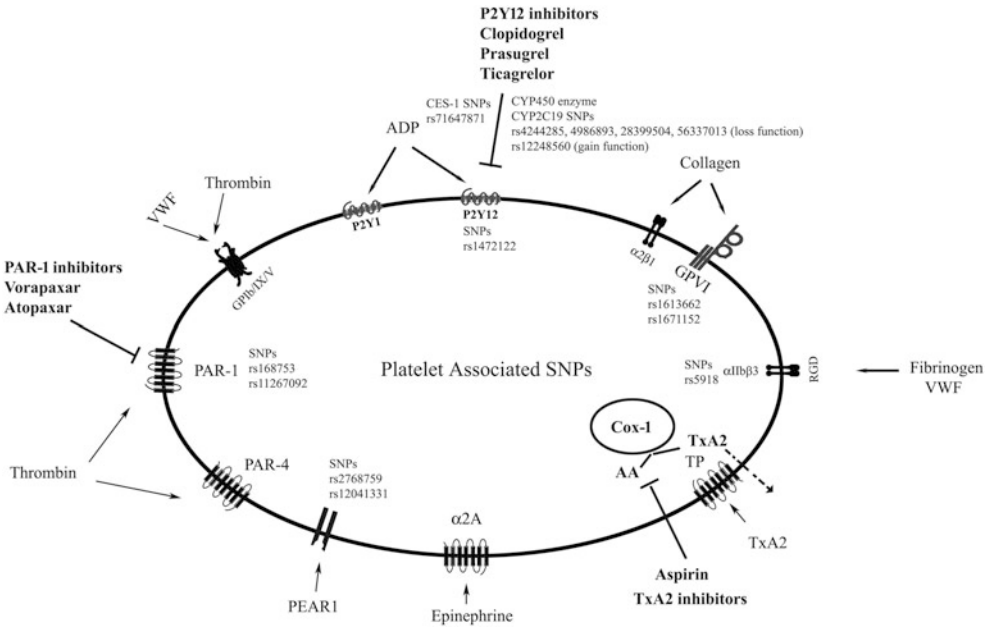

Fig. 2 SNPs studied in antiplatelet drug response. SNPs of platelet receptors and SNPs of enzymes involved in antiplatelet drug metabolism have been studied for the correlation with antiplatelet drug response and some of these SNPs are critical for individual differences of antiplatelet drug response

of 86 % (H1) and 14 % (H2), respectively, have been reported in healthy population (Fontana et al. 2003). In addition, the H2 haplotype was associated with increased prevalence of coronary and peripheral artery disease (CAD, PAD) (Cavallari et al. 2007). In another study, 5 polymorphisms in the P2Y1 gene and 11 in the P2Y12 gene have also been identified (Hetherington et al. 2005). However, in a genome-wide study, none of these polymorphisms were associated with defective platelet function except the *rs1472122* SNP (Jones et al. 2009).

Collagen Receptors

The integrin $\alpha_2\beta_1$ and the platelet-specific receptor GPVI are two major collagen receptors in platelets (Fig. 2). There are three major alleles encoding for the α2 chain; high level expression of α2β1 has been associated with allele 1 (807T/1648G/2531C) while decreased expression of the receptor is associated with other two alleles (allele2: 807C/1648G/2531C; allele 3: 807C/1648A/2531C) (Kunicki et al. 1997). In addition, the 807T allele has been associated with acute myocardial infarction (MI) risk (Santoso et al. 1999; Moshfegh et al. 1999). To date, there is no convincing evidence for thrombotic risk in cohorts associated with polymorphisms of β1 subunit (*ITGB1*). For variants of *GPVI*, the *GPVI* 13254CC genotype has been associated with increased risk of MI and thrombus formation (Croft et al. 2001; Ollikainen et al. 2004). In genome-wide functional studies, the variants *rs1613662* and *rs1671152* have been associated with agonist-induced P-selectin expression, fibrinogen receptor binding, and collagen-induced platelet aggregation, respectively (Jones et al. 2009; Johnson et al. 2010).

Fibrinogen Receptor

Integrin $\alpha_{IIb}\beta_3$ is the major fibrinogen receptor on platelets (Fig. 2). To date, the most intensely studied polymorphic variant of GPIIIa is SNP *rs5918* (Floyd and Ferro 2012). Nonetheless, the role of this variant in innate platelet reactivity and in cardiovascular diseases leads to inconsistent result. While initial studies with small cohort sizes suggested that GPIIIa gene polymorphism may be associated with the risk for developing coronary artery disease and coronary/cerebrovascular thrombosis, subsequent meta-analyses with combined cohort sizes have not been confirmatory (Michelson et al. 2000; Aalto-Setala et al. 2005; Slowik et al. 2004; Ye et al. 2006).

Platelet Genome-Wide Association Studies (GWAS)

Previous candidate gene analyses have mainly focused on genes considered to be important in the function of platelets (a priori) and/or hemostatic disorders, with shortcomings in large-scale studies (Chami and Lettre 2014). Since the majority of platelet SNPs with minor allele frequencies (MAF) (<0.2) and most platelet phenotypes have a small effect size with high interindividual variability, large cohorts and genomic technologies are required to detect rare variants and to elucidate their effects with sufficient statistical power in multivariate analyses and multiple testing to minimize the false discovery rate (FDR). With the advent of next-generation/high-throughput DNA/RNA sequencing, the identification of novel genes associated with platelet dysfunction and thrombocytopenia has become more common. Many new

causes of familial thrombocytopenia and platelet dysfunction have been described in the past few years using these methods (Bunimov et al. 2013; Chami and Lettre 2014). We now discuss recent GWAS with three aspects of platelet biology: platelet volume, platelet count, antiplatelet drug responses, etc.

GWAS: Platelet Counts and Volume

Mean platelet volume (MPV) and platelet count (PLT) are highly heritable and tightly regulated traits. Platelet volume is a marker of platelet function and activation (Azab et al. 2011; Chu et al. 2010; Gasparyan et al. 2011; Lance et al. 2012; Sansanayudh et al. 2014; Vizioli et al. 2009). MPV is increased in certain vascular risk factor states, including hypercholesterolemia and diabetes mellitus, but not essential hypertension. It is increased in acute myocardial infarction, acute ischemic stroke, preeclampsia, and renal artery stenosis. Importantly, an elevated MPV predicts a poor outcome following myocardial infarction, restenosis following coronary angioplasty, and the development of preeclampsia (Bath and Butterworth 1996). Different genetic variants have been associated with MPV and platelet count using candidate gene subsets. Recently, a number of GWAS studies have been reported focusing on these critically important parameters (Table 2).

In one report, 49,094 single-nucleotide polymorphisms (SNPs) that capture variation in ~2100 candidate genes in DNA of 23,439 Caucasians and 7112 African Americans have been studied and strong novel associations between platelet count and a SNP at the tropomyosin-4 (*TPM4*) locus (*rs8109288*) have been found. The data suggests similarities but also differences in the genetic regulation of hematological traits in European- and African-derived populations (Lo et al. 2011). In another GWAS study, it was shown that MPV was strongly associated with three common SNPs: *rs7961894* located within intron 3 of *WDR66* on chromosome 12q24.31, *rs12485738* upstream of *ARHGEF3* on chromosome 3p13-p21, and *rs2138852* located upstream of *TAOK1* on chromosome 17q11.2 and expression analysis indicated a direct correlation of WDR66 transcripts and MPV (Meisinger et al. 2009).

A different GWAS for MPV identified one SNP *rs342293* as having highly significant and reproducible association with MPV in 8586 healthy subjects. Whole-genome expression analysis found that it had a significant association with platelet transcript levels for *PIK3CG*. In addition, the G allele at *rs342293* was also associated with decreased binding of annexin V to platelets activated with collagen-related peptide. The region 7q22.3 is correlated with platelet volume, counts, and function in healthy subjects (Soranzo et al. 2009a). Another GWAS reported 15 out of 22 common variants in 13,943 samples from 6 European population-based studies associated with platelet parameters including MVP and counts (Soranzo et al. 2009b). In a GWAS involving 14,700 Japanese individuals, 5 out of 60 associations are correlated with platelet counts (Kamatani et al. 2010). The largest platelet GWAS to date involves approximately 67,000 individuals with a European background. In this study, 68 independent genomic regions have been identified and that were related to MPV and platelet count (25 and 43 regions for MPV and platelet count, respectively). The 68 reported loci account for about 5 % of variability of platelet count and 10 % of variability of MPV. Replication of selected loci was obtained in cohorts with different ethnic background (Gieger et al. 2011).

In summary, data from various groups and cohorts have identified various SNPs and gene loci to be associated with platelet counts and MPV. However, SNPs and genetic variants identified by one group have not been consistently identified by other groups (Shameer et al. 2014; Oh et al. 2014), limiting firm conclusions relevant to pathogenesis or molecular regulation of MPV; clearly, larger cohorts with more robust controls and molecular validation studies will be required for further insight into molecular regulatory signals controlling platelet size, and how these variants may control functional platelet responses and clinical outcomes (Table 2).

Table 2 GWAS: platelet count and mean platelet volume (MPV)

Cohort size	Platelet count	MPV	Genomic loci	Reference
66,867	56 SNPs	29 SNPs	68	Gieger et al. (2011)
23,439 Caucasians	Caucasian: rs8109288		Caucasian: 1	Lo et al. (2011)
7112 African Americans	African American: rs10876550; rs8109288		African American: 2	
10,048		rs7961894; rs12485738; rs2138852	3	Meisinger et al (2009)
8586		rs342293	1	Soranzo et al. (2009a, b)
13,943	rs11065987; rs11066301; rs210135; rs385893	12 SNPs	16	Soranzo et al. (2009a, b)
14,700	rs739496; rs7775698; rs385893; rs6141; rs5745568		5	Kamatani et al (2010)

GWAS: Antiplatelet Drug Response

The effect of genetic variants on antiplatelet drug responses has been studied for commonly used agents including aspirin, clopidogrel, and prasugrel (Fig. 2). Not surprisingly, these GWAS studies have the same restrictions for large cohort sizes due to small SNP effects and the presence of low-frequency SNP alleles (Meyer et al. 2013; Geisler et al. 2013; Perry and Shuldiner 2013; Zuern et al. 2010; Sabatine and Mega 2014).

Aspirin

The correlation between aspirin resistance and the polymorphisms of cyclooxygenase 1 (*COX-1*), cyclooxygenase 2 (*COX-2*), as well as platelet integrin GPIIIa has been studied with controversies (Freedman 2006; Faraday et al. 2007a, b; Frelinger et al. 2006; Cui et al. 2015). However, SNPs of platelet endothelial aggregation receptor 1 (PEAR1) have been identified in functional genomic and genome-wide association studies. It has been shown that the C allele of *rs2768759* [A/C] located in the promoter region of this gene was associated with lower responsiveness to aspirin measured by collagen- and epinephrine-induced platelet aggregation (Herrera-Galeano et al. 2008). In addition, variant *rs12041331* in intron 1 has been found to account for up to 15 % of variation of platelet function and was strongly linked with agonist-induced platelet aggregation (collagen, epinephrine, and ADP) at baseline and during aspirin treatment (Faraday et al. 2011; Kim et al. 2013; Lewis et al. 2013b).

Clopidogrel

Two steps are required to convert clopidogrel to its biologically active thiol metabolite. The first step, a monooxygenation reaction of the thiophene ring by CYP450 enzymes (i.e., *CYP2B6*, *CYP2C19*) leads to 2-oxo-clopidogrel, a thiolactone metabolite. The second step results in a CYP450-dependent (i.e., *CYP2B6*, *CYP2C19*) oxidative opening of the thiolactone ring of 2-oxoclopidogrel (Sangkuhl et al. 2010). *CYP2C19* is highly polymorphic and the variations include the loss-of-function alleles *CYP2C19* *2,*3,*4, and *5 and the ultra-rapid metabolizer allele *CYP2C19*17 (Zanger et al. 2014; Zanger and Schwab 2013). The effect of *CYP2C19* polymorphisms on the antiplatelet activity of clopidogrel has been studied and a significant impact of *CYP2C19* loss-of-function alleles on enhanced platelet aggregation has been found in clopidogrel-treated patients (Simon et al. 2009; Geisler et al. 2008). Subsequent studies established that patients carrying one or more *CYP2C19* loss-of-function allele (s) have had higher rates of cardiovascular events than noncarriers (Hulot et al. 2006; Trenk et al. 2008; Mega et al. 2009). In a GWAS study, it was confirmed that SNPs on chromosome 10q24 encompassing the *CYP2C18–CYP2C19–CYP2C9–CYP2C8* gene cluster were strongly associated with decreased clopidogrel response. The *rs12777823* variant was nearly in complete linkage disequilibrium with the *CYP2C19*2* variant, accounting for up to 12 % of variability of on-treatment platelet aggregation (Shuldiner et al. 2009).

Carboxylesterase 1 (CES-1) is another enzyme involved in the drug metabolism of clopidogrel. It is involved in the conversion of clopidogrel, its intermediate 2-oxo-clopidogrel, and the final bioactive thiol metabolite into biologically inactive carboxylic acid derivatives. The *G143E* (rs71647871) genetic variant is linked to enzyme function and a recent report has shown a significant association of the *CES-1* G143E SNP allele with higher active metabolite levels and higher inhibition of ADP-induced platelet aggregation (Lewis et al. 2013a). These positive results contrast with those assessing the role of the arylesterase paraoxonase (*PON1*) in clopidogrel bioactivation. In one study, a *PON1* polymorphism (rs662) demonstrated no significant correlation with clopidogrel response (Reny et al. 2012). In addition, the correlation between allelic variants of two distinct platelet receptors (H2 haplotype of *P2Y12* and *PAR1*) and clopidogrel platelet response has also been studied with no reproducible correlation with either allelic system (Angiolillo et al. 2005; von Beckerath et al. 2005; Motovska et al. 2009).

Prasugrel

In contrast to clopidogrel, *CYP450* enzymes are involved in a single step of prasugrel metabolism with a minor contribution from *CYP2C19*. Plasma levels of the active metabolite were not influenced by common genetic variants of *CYP2C19, CYP2C9, CYP2B6, CYP3A5,* or *CYP1A2* (Mega et al. 2009). Recent studies show a significant impact of *CYP2C19* polymorphisms (*2 and *17) on platelet reactivity and the bleeding risk during chronic treatment with prasugrel was influenced by *CYP2C19* genetics (Cuisset et al. 2012). Another study suggests that both clopidogrel and prasugrel therapy may be altered by the *CYP2C19* *2 and *17 alleles (Grosdidier et al. 2013). Taken together, further studies will be needed to definitely establish the contribution of CYP450 genetic variants in prasugrel bioactivation and pharmacokinetic responses.

Ticagrelor

Ticagrelor is primarily metabolized by *CYP3A4/5* enzymes, and *CYP2C19* does not seem to play a relevant role in drug metabolism. To date polymorphisms involving *CYP2C19, ABCB1, P2Y12, P2Y1*, and *ITGB3* demonstrate no consistent effects on inhibition of ADP-induced aggregation by Ticagrelor (Zhou et al. 2011; Tantry et al. 2010; Wallentin et al. 2010).

Thrombin Receptor (PAR1) Antagonists

A limited number of polymorphisms of the *PAR1* receptor gene have been reported in clinical studies. The intervening sequence intronic variation *rs168753* is associated with reduced PAR-1 receptors on the platelet surface and with the response to agonist stimulation; the insertion/deletion variant *rs11267092* has been evaluated for its protective role in venous thromboembolism (Dupont et al. 2003; Arnaud et al. 2000). However, none of these variants have been associated with clinical efficacy of PAR1 antagonist vorapaxar or atopaxar yet.

GWAS: Platelet-Associated Diseases

To date the most intensively studied platelet-associated disease using GWAS is myocardial infarction where platelets play a pivotal role in atherothrombosis (Kathiresan et al. 2009; Ouwehand 2007, 2009). GWAS have also been performed in cohorts with stroke and major depressive disorder (MDD) (Ziegelstein et al. 2009; Wray et al. 2012).

Myocardial Infarction

Platelets play a pivotal role in atherothrombosis after coronary artery plaque rupture, and the extent of platelet response during such an event varies between individuals. To some degree, this variability appears to be genetically controlled. Evidence that the megakaryocyte DNA content correlates with coronary artery atherosclerosis was first suggested by Bath et al. about two decades ago (Bath et al. 1994; Dalby Kristensen et al. 1988). More comprehensive GWAS to correlate different gene loci with MI risk have identified the first 11 risk loci for myocardial infarction (MI) and coronary artery disease (CAD). Studies on quantitative traits provide an alternative approach to identify MI/CAD risk loci (Ouwehand 2007, 2009; Mathias et al. 2010). Nonetheless, specific gene identification, validation, and precise molecular mechanisms for these associations remain ongoing.

Stroke

In a meta-analyses GWAS involved 14,746 African Americans (1365 ischemic and 1592 total stroke cases), genetic variants were examined for validation in METASTROKE and nominal associations ($p < 10^{-6}$) for total or ischemic stroke were observed. Of note, one of these variants (*HPS4*) is implicated in the protein component regulating biogenesis of lysosome-related organelles complexes (BLOC). BLOC complexes are important for the formation of endosomal–lysosomal organelles such as melanosomes and platelet dense granules, and are causally implicated in Hermansky–Pudlak Syndrome (Carty et al. 2015).

Major Depressive Disorder

MDD is a common, complex disorder with a partial genetic component. Adenylyl cyclase (AC) is an enzyme that can regulate the physiologic effects of numerous drugs and hormones through the production of cyclic adenosine-3′, 5′-monophosphate (cAMP). The relationship between platelet AC activity and lifetime diagnosis of major depression among 1481 participants (226 subjects with a history of major depression and 1255 control subjects) has been assessed and it was found that a lower platelet AC activity was associated with MDD (Hines and Tabakoff 2005). However, in another genome-wide association study, no SNPs have been found significantly associated with MDD although gene-based tests suggested a role for adenylate cyclase 3 (ADCY3, 2p23.3) in development of MDD (Wray et al. 2012).

Conclusion and Future Directions

GWAS is a robust and powerful approach to identify novel genes and pathways involved in platelet function under normal or pathological conditions. Although data studying the role of genetic polymorphisms with platelet function and or efficacy of antiplatelet agents is not always consistent, a unifying concept forming a multitude of studies strongly suggest that variability of platelet activation largely depends on genetic inheritance and variability. While many SNPs have been identified and linked to platelet function, delineation of SNP–SNP interactions for optimal gene/phenotypic studies remains an area of critical need and investigation. Furthermore, genetic variations of noncoding regions which incorporate transcription factor binding sites, etc., and epigenetic modifications (such as DNA methylation, histone acetylation, etc.) need to be incorporated into future studies (Freson et al. 2012). Integration of large data sets

incorporating clinical phenotype, functional assays, and high throughput data such as genome sequencing, RNAseq, microarray, and proteomic studies demands advanced bioinformatical solutions (De et al. 2014). In addition, a robust algorithm in statistical studies needs to be developed to prevent misleading false discovery. In theory, these issues should be addressed using integrated systems biology approaches, thereby providing optimal geno/phenotypic data linked to optimal outcomes and tailored antiplatelet interventions.

Take Home Messages

1. Genetic variability of both intronic and exonic sequences is implicated in regulation of platelet reactivity, volume, peripheral counts, and cellular responsiveness. Many genetic variants have subtle effects and a single subtle effect may be difficult to delineate or consistently reproduce across cohorts.
2. Genome-Wide Association Study (GWAS) is a robust approach to identify novel genes and pathways involved in platelet function under normal or pathological conditions. GWAS with large cohort sizes and well-defined phenotypes could provide valuable information to identify new genetic variations with subtle effect(s) on platelet functional parameters.
3. While many SNPs have been identified and linked to platelet function, delineation of SNP–SNP interactions for optimal gene/phenotypic studies remains an area of critical need and investigation. Genetic variations of noncoding regions which incorporate transcription factor binding sites, etc., and epigenetic modifications (such as DNA methylation, histone acetylation, etc.) need to be incorporated into future studies. In addition, a better algorithm in statistical studies needs to be developed to prevent misleading false discovery.

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The Platelet Transcriptome: Coding RNAs

Jesse W. Rowley, Bhanu Kanth Manne, and Andrew S. Weyrich

Abstract

Until recently platelets were categorized as simple anucleate cytoplasts that function solely in the hemostatic arena. However, the last decade has demonstrated that platelets have roles beyond primary hemostasis and they possess complex molecular information including a rich repertoire of messenger RNAs (mRNAs). Platelet mRNAs have features that mirror transcripts produced and translated in nucleated cells, and mRNAs for approximately half of the human genome have been identified in this terminally differentiated cell. The study of platelet mRNAs has expanded tremendously with the advent of next-generation RNA sequencing (NGS) and several groups have shown that platelets are capable of translating some megakaryocyte-derived mRNAs into protein. Platelet mRNA profiles have also been used to predict cellular function and platelets are capable of transferring intact mRNAs into microparticles and other cells. This chapter reviews our current understanding of platelet mRNAs and their potential contribution to biological and pathologic processes in humans. Roles for noncoding RNAs are described in Edelstein and Bray (2017).

General Overview of the Platelet mRNA World

Although platelets have been studied for over a century, it wasn't until the late 1980s that Newman and colleagues used PCR-based studies to identify mRNA for GPIIIa in platelets (Newman et al. 1988). These studies set the stage for direct molecular characterization of proteins in healthy subjects and patients with inherited platelet disorders. The subsequent development of microarrays and Serial Analyses of Gene Expression (SAGE) allowed for a broader analysis of mRNAs in platelets. Studies using these techniques revealed that mitochondrial-coded RNAs are abundant in platelets, and that platelets express messages that code for proteins involved in hemostasis, thrombosis, and inflammation (Gnatenko et al. 2003; Dittrich et al. 2006). These studies also showed that platelet mRNAs have long untranslated regions (UTRs) and that messages coding for cytoplasmic polyadenylation elements (CPEs), mRNA localization, and translational control are enriched in the platelet transcriptome (Dittrich et al. 2006).

Microarray, SAGE, and other hybrid-based techniques have recently been supplanted by NGS (Shendure 2008) that enables unprecedented characterization and quantification of the platelet transcriptome (Mortazavi et al. 2008; Costa et al. 2010). Several groups have now employed NGS and shown that most proteins have a corresponding mRNA expressed in human platelets (Rowley et al. 2011; Kissopoulou et al. 2013; Bray et al. 2013; Rowley and Weyrich 2013; Londin et al. 2014; Osman et al. 2015; Cimmino et al. 2015; Eicher et al. 2016; Best et al. 2015). A little over 40 % of mapped long RNA-seq reads correspond to mRNAs of protein coding genes, 32 % correspond to rRNA, and only 6 % map to annotated nonprotein coding loci (Londin et al. 2014). Thus, the majority of the long RNAs (defined as >200 bp, but reads are often detected at >100 bp) in the cell are involved in protein translation. Of

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the reads mapping to protein coding genes, the majority map to a handful of genes coding for mitochondrial proteins (Bray et al. 2013), and the remainder map to 7000–10,000 different nuclear-encoded protein-coding genes (Rowley et al. 2011; Bray et al. 2013). The distribution of expression of these transcripts matches that of other cells (Fig. 1). Therefore, the platelet mRNA-ome is complex (Rowley et al. 2011), with more than half of the known human protein coding genes represented in platelets. The remainder of long RNA reads in platelets match to lincRNAs, unannotated intronic and intergenic regions, pseudogenes, and a variety of repeat elements (Londin et al. 2014). Using PTESFinder Pipeline v. 1 (http://sourceforge.net/projects/ptesfinder-v1/), Alhasan and colleagues (2016) have also recently shown that platelets are enriched in noncoding circular RNAs (circRNA), which are resistant to exonuclease degradation. The precise biological/pathologic function(s) of circRNAs in eukaryotic cells is unclear, but focused investigations over the coming years should provide insight into the roles of circRNAs in platelets. Short RNAs (17–200 bp), such as miRNAs, that are detectable by short-RNA sequencing, are also abundant in platelets (Plé et al. 2012a). Short RNAs and other noncoding RNAs are discussed in a subsequent chapter.

As recently reviewed elsewhere (Schubert et al. 2014), the majority of platelet-coding mRNAs appear to be mature

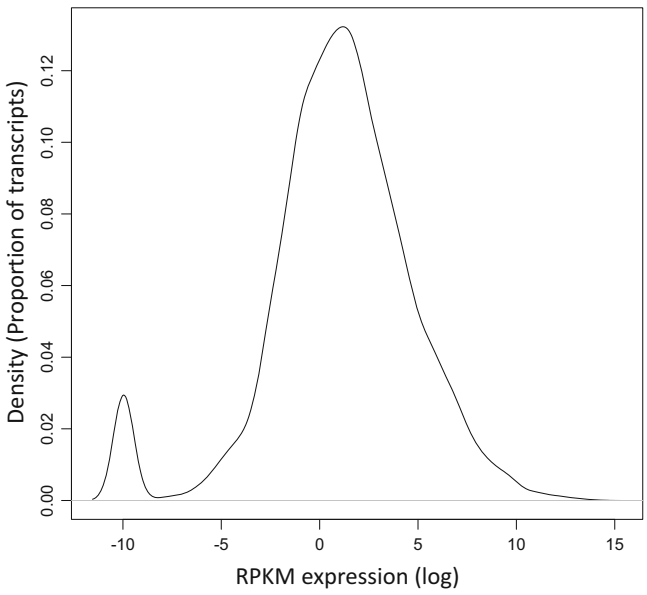

Fig. 1 Distribution of mRNA expression levels in platelets. The histogram depicts the relative levels (on a log scale) of all mRNAs in platelets as measured by RNA-sequencing of poly-A RNA. Note that the distribution is bimodal, similar to what has been described in other cells (Nagaraj et al. 2011). There is a secondary peak of very low abundant transcripts, but the majority of the transcripts follow a normal distribution of expression with a range of expression from low to very abundant

mRNA which are spliced (free of introns), capped, and polyadenylated at their 5′- and 3′-UTRs, respectively. Evidence of capping and polyadenylation comes on several fronts, including deep sequencing data captured by cap-binding proteins (Choi and Hagedorn 2003) or oligo-DT conjugated beads that target poly-A tails (Rowley et al. 2011). Consistent with these features, platelet mRNAs are competent for protein coding when placed in in vitro translation systems and incorporate labeled amino acids into a range of proteins in vivo (Warshaw et al. 1967; Booyse and Rafelson 1967; Shaw et al. 1984; Kieffer et al. 1987). Nevertheless, thus far only 10–20 of the thousands of mRNAs have been specifically demonstrated to be translated in a constitutive or signal-dependent fashion in human platelets (Weyrich et al. 2009; Rowley et al. 2012), including the mTOR-dependent translation of B-cell lymphoma 3 (Bcl-3) following platelet activation (Weyrich et al. 1998). Translation of Bcl-3 mRNA and other messages, including pre-mRNAs for IL-1β (Denis et al. 2005) and Tissue Factor (Schwertz et al. 2006) that are spliced and translated in a signal-dependent fashion, are reviewed elsewhere (Rowley et al. 2012).

The Inheritance of Platelet mRNAs

It is generally believed that most platelet mRNAs are derived from their parent megakaryocytes. Steady-state levels of platelets are maintained by production of new platelets from megakaryocytes and destruction of platelets in the liver and spleen (Mason et al. 2007). Before a megakaryocyte gives birth to platelets, a massive increase in cell growth and DNA synthesis occurs resulting in “mega” cells up to 128 N through repeated endomitotic divisions (Zimmet and Ravid 2000). During the final stages of development, megakaryocytes produce platelets by protruding or fragmenting their cytoplasm (Junt et al. 2007), or during situations of acute platelet need, by rupture (Nishimura et al. 2015). mRNAs are transcribed during the entire process of megakaryocyte development (Raslova et al. 2003), and those transcripts that eventually reach platelets, depending on their stability, potentially come from all stages of megakaryocyte maturation. In the megakaryocyte, as with other cells, precursor RNA is transcribed by RNA-polymerase II. Nascently transcribed RNA is cotranscriptionally loaded (Darnell 2013) with nuclear protein factors that include splicing factors, export signals, and zip code-binding proteins (Buxbaum et al. 2014), for splicing of precursor mRNA into mature mRNA, export from the nucleus (Delaleau and Borden 2015), and trafficking into platelets. Because transcription aborts at or prior to platelet release, gradual loss of mRNA occurs (Alhasan et al. 2016). Young platelets are thought to have more mRNA (Chavda

et al. 1996), increased numbers of ribosomes, and higher synthetic potential (Steiner and Baldini 1969).

Mechanisms by which mRNAs get transferred from megakaryocytes to platelets are unknown. However, there is evidence that mRNAs are differentially sorted rather than randomly distributed into platelets. In this regard, Cecchetti et al. (2011) have shown that matrix metalloproteinase (MMP) and tissue inhibitors of metalloproteinase (TIMP) family members are differentially packaged into platelets. By example, MMP-2 protein is in platelets but its transcript is only found in the megakaryocyte, whereas Timp-2 protein is very low in platelets but the mRNA is abundant. Studies of additional targets and on primary bone marrow or tissue megakaryocytes will help to clarify whether dedicated processes such as zip code-binding proteins direct the majority of mRNA into proplatelets, whether RNA is captured based on its peripheral location, or whether most are simply randomly selected. Attention will also need to be given to transfer of mRNA into platelets to and from other blood cells. Platelets are adept at endocytosis, and numerous investigations have shown that horizontal RNA transfer occurs between cells, including transfer of platelet mRNA to endothelial cells and hepatocytes (Tang et al. 2010; Risitano et al. 2012; Laffont et al. 2013; Clancy and Freedman 2014).

NGS Overview

There are a variety of approaches to measure the expression, individually or globally, of platelet mRNAs. NGS is currently the preferred method of transcriptome-wide studies because it unbiasedly captures a snapshot of mRNA presence, structure, and quantity from a genome at a defined moment in time (Shendure 2008). Here we briefly describe NGS but refer the reader elsewhere to more detailed reviews of this technology and its application to platelets (Wang et al. 2009; Costa et al. 2010; Auer and Doerge 2010; Nekrutenko and Taylor 2012; Schubert et al. 2014).

RNA-sequencing studies include four main steps: RNA isolation, Library Preparation, Sequencing, and Bioinformatic alignment and analysis of expression (Fig. 2). For platelet RNA, common isolation methods including phenol/chloroform-based extraction (TRIzol) or other commercial RNA isolation kits that allow for low abundance RNA isolation are suitable. After total RNA isolation, an RNA selection step targeting the class of RNA of interest is performed. For example, oligo-dT is commonly used to select polyadenyated mRNAs of coding genes. Other strategies, each with their own advantages and disadvantages, include ribosomal RNA removal, pull-down

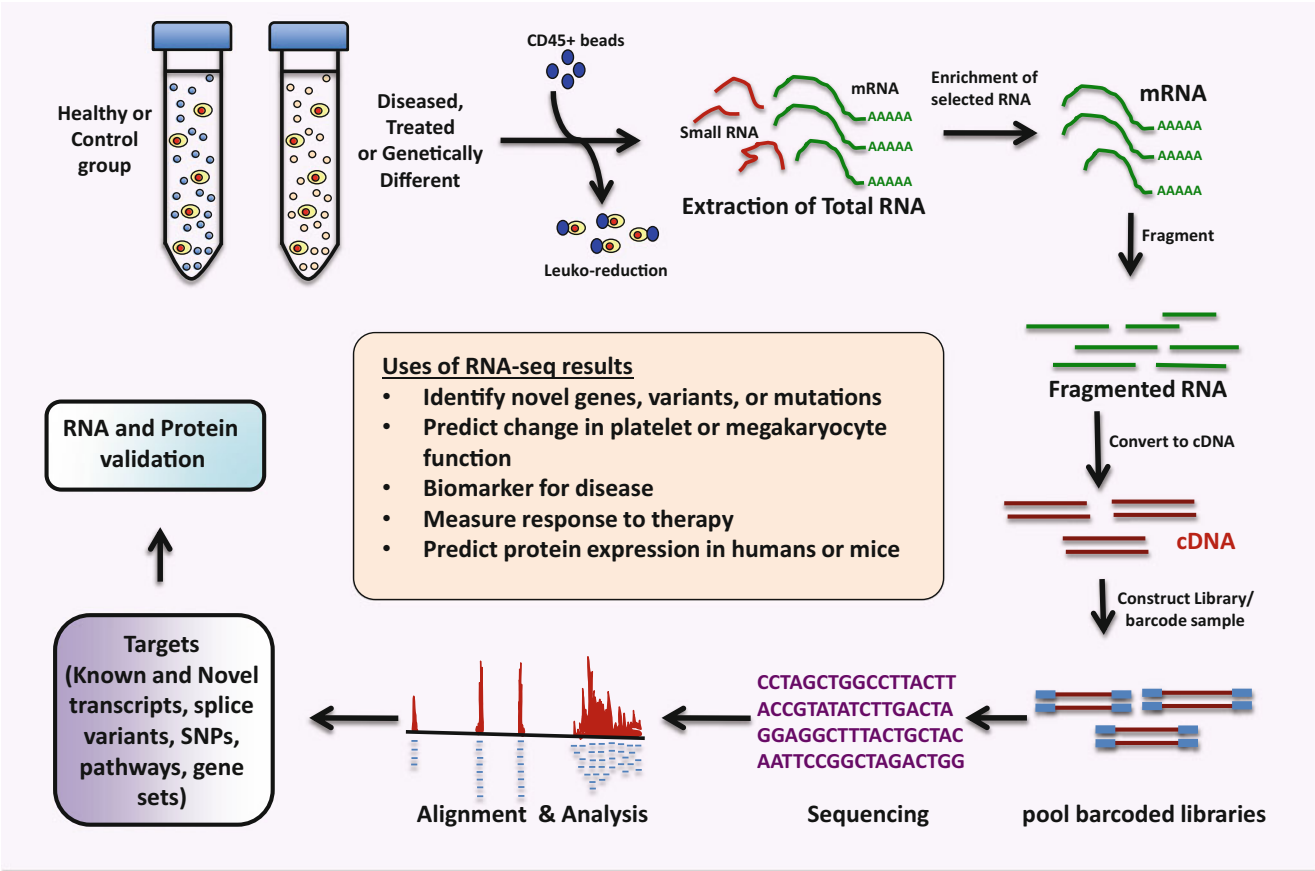

Fig. 2 A schematic overview of the typical workflow for RNA-sequencing studies in platelets

of capped RNA, or capturing of mRNAs associated with a specific RNA-binding protein.

Library preparation procedures vary according to the RNA species, desired analysis, and sequencing technology employed, but often include RNA fragmentation and size selection, adapter addition, cDNA generation, and amplification. Libraries can be prepared to maintain strand information, and therefore both sense and antisense transcripts can be identified from the same RNA pool. Barcodes are often added so that multiple libraries (we commonly pool >8 on an Illumina Hiseq 2000) can be pooled and distinguished bioinformatically. Following library prep, fragments are sequenced by available chemistries and platforms which include Illumina, Roche 454, SOLiD, Ion Proton/PGM, or Pac-bio sequencing. Fragment sequences are processed into the 4 letter nucleic acid code, and each sequence is either aligned to the genome, to the transcriptome, or assembled de novo into a transcriptome (Martin and Wang 2011).

Relative mRNA abundance, or the abundance of mRNA features (splice junctions, UTRs, individual exons or isoforms, etc.), can be quantified by counting the number of reads aligning to mRNA, followed by a normalization step. Estimates of expression based on read counts, RPKMs or FPKMs (reads or fragments per kilobase normalized to a million mapped reads) (Mortazavi et al. 2008), or normalization to reference transcripts such as ActB (Londin et al. 2014) are still commonly employed, although more sophisticated normalization techniques can give more accurate estimates of expression and are preferred for more rigorous statistical analysis (Wagner et al. 2012; Dillies et al. 2013).

As investigators embark on NGS-based studies, they need to keep in mind that bottlenecks can occur at the stage of data analyses. Thus, having a bioinformatics pipeline in place or access to collaborators with experience in managing NGS data is critical. Leukocytes contain >10,000 times more mRNA per leukocyte than a platelet (Fink et al. 2003). Thus, the sensitivity of NGS to detect mRNAs of low expression also requires stringent removal in platelet preparations of leukocytes that can confound data interpretation. Follow-up studies of candidate mRNAs and/or their corresponding protein are usually required to prove the expression and functionality of the target gene in platelets.

Platelet Transcripts as Indicators of Altered Platelet Function

Environmental and genetic factors influence the functional response of platelets. With regards to the environment, aging (Mohebali et al. 2014), diet (McEwen 2014), stress (Suls 2013), exercise (Heber and Volf 2015), obesity (Bhatt 2008; Anfossi et al. 2009; Russo et al. 2010), diabetes (Bhatt 2008), infection (Assinger 2014; De Stoppelaar et al. 2014), heart disease (Béres et al. 2008), immune or inflammatory disease (Herter et al. 2014) are known to alter platelet functional responses. For several of these conditions, platelet mRNA has been used to understand the mechanistic causes of altered platelet function or determine how differences in the platelet transcriptome might influence disease. Because their coded proteins, rather than the mRNAs themselves, are the executors of platelet function, mRNA is used as a surrogate to identify altered proteins, or in some cases to identify altered pathways. This is possible because the mRNA and protein levels in platelets are correlated. A comparison between platelet mRNA measured by RNA-seq and protein levels measured by quantitative proteomics has recently demonstrated this correlation (Rowley and Weyrich 2013). While the extent of the protein–mRNA relationship in platelets is still under debate (Geiger et al. 2013; Londin et al. 2014), the correlation is strong enough to have allowed many groups to successfully validate protein and pathway changes originally identified from mRNA screening. Some examples are highlighted.

CD69 and MRP 8/14 mRNA levels discriminated between STEMI and coronary artery disease (Healy et al. 2006). Protein levels of Myeloid Related Protein 8/14 mRNA correlated with the mRNA level and higher levels of MRP 8/14 predicted cardiovascular risk independent of other risk factors. Later, it was shown that MRP 8/14 is associated with thromboxane-dependent activation (Santilli et al. 2014). Genetic knockout of MRP 8/14 in mice prolonged the time to arterial occlusion, and transfusion of MRP 8/14 sufficient platelets into MRP 8/14 deficient mice decreased time to occlusion (Wang et al. 2014).

Pathway analysis of differentially expressed transcripts in sickle cell disease successfully predicted increased arginase II activity in platelets from diseased individuals (Raghavachari et al. 2007). In another related study, mRNA protein levels of inflammatory cytokines were profiled in patients with sickle cell disease. mRNA and protein for IL-1β, sCD40L, and IL-6 were increased in disease compared to controls (Davila et al. 2015). Lood and colleagues discovered an IFN response signature in the platelet transcriptome, with coordinate protein changes in IFN-regulated proteins such as PRKRA, IFITM1, and CD69 from patients with systemic lupus erythematosus (SLE) (Lood et al. 2010). In addition, COMMD7 and LRRFIP1 were identified as transcripts associated with platelet responsive to ADP or CRP-XL as measured by flow cytometry. When tested for replication of genotyping, LRRFIP1 and COMMD7 harbored single nucleotide polymorphisms (SNPs) potentially associated with MI (Goodall et al. 2010). The functionality of these genes was tested in a laser injury model in zebra fish where Lrrfip1-deficient

zebra fish exhibited a longer time to occlusion and a smaller thrombus size following thrombus induction (Goodall et al. 2010).

Environmentally induced mRNA expression changes in platelets have been identified in several other studies that lack corresponding protein data. Pre- and post glucose infusion studies identified altered insulin response pathways (Rao et al. 2014) and potential differences in mRNA candidates were identified in platelets from STEMI versus non-STEMI individuals (Eicher et al. 2016). Microarray analysis of platelets was also able to distinguish classes of thrombocytosis (Gnatenko et al. 2010) and RT-PCR of mitochondrial transcripts demonstrated an increase in mitochondrial complex I transcripts in older individuals (Merlo Pich et al. 2004). mRNA profiling has been used to identify candidate mRNAs altered in platelets from chronic kidney disease patients where changes in transcript expression were corrected by dialysis (Plé et al. 2012b). One of the altered transcripts was PCTP, an mRNA newly implicated in platelet function (Edelstein et al. 2013) (see later). Differences in these biomarker-based studies set the stage for subsequent examination of protein levels that will help clarify whether the observed changes in transcript expression are linked to altered functional responses in platelets.

Coding mRNA and Genetic Differences in Platelet Function

Genetic differences in the promoters, UTRs, introns, or coding region can influence the mRNA/protein expression level and the function of the product coded by platelet mRNAs. Genetic differences, if found within exons of mRNA are readily detected by RNA-seq (Kahr et al. 2011; Noetzli et al. 2015). Inherited common SNPs, race/ethnicity, gender, and pathological mutations are genetic factors that alter the mRNA content in platelets and dictate platelet function.

One study correlated the expression of Vamp8/endobrevin with platelet reactivity (Kondkar et al. 2010). The level of Vamp8 was related to a SNP in the 3′-UTR thought to alter miRNA binding and regulation of Vamp8 expression. Since Vamp8 is involved in platelet release, this provided a mechanistic explanation to some of the variability observed in platelet reactivity.

To identify transcripts related to racial differences in platelet reactivity, platelet functional responses and platelet mRNA were profiled from 70 black and 84 white subjects in the PRAX-1 study (Edelstein et al. 2013; Simon et al. 2014). Platelet responses to Par4 were higher in blacks than whites, and according to microarray analysis, PCTP mRNA is fourfold higher in blacks than in whites. Differences in PCTP could be attributed to lower miR-376c in blacks. PCTP protein levels were also higher. The role of PCTP protein in potentiating platelet reactivity was further supported by PCTP inhibition and depletion studies. Other relationships between platelet transcript levels; miRNA levels; and a number of demographic, platelet function, and clinical parameters collected as part of the PRAX-1 study (Platelet RNA AND eXpression 1 study) (Simon et al. 2014) can be explored at www.plateletomics.com. For example, the PRAX-1 study identified 129 transcripts differentially expressed by age in platelets and 54 by gender. In addition to age, race, and gender, transcript relationships with BMI; fibrinogen levels; von willebrand factor (VWF); and functional associations with ADP, arachidonic acid, Par4 AP, and Par1 AP can be explored to generate testable hypotheses.

mRNA studies have also been used to identify mutations leading to pathologic changes related to platelet function. Alterations in VWF splicing caused by a synonymous mutation within splicing regulatory regions could not be predicted without careful analysis of mRNA (Pagliari et al. 2013). In this study, mRNA analysis of three unrelated patients with von willebrand disease (VWD2AIIE) uncovered a 159 bp deletion (exon 26) in the mRNA that was not apparent from DNA sequencing. The patients shared a synonymous single nucleotide non-splice site substitution that affected splicing. Other splice site mutations leading to exon skipping in VWD have also been identified (Yadegari et al. 2012; Pagliari et al. 2013).

NGS has also been used to facilitate the discovery and understanding of rare genetic disorders that affect platelet function. Specifically, sequencing of platelet mRNA from patients with Gray Platelet Syndrome (GPS) detected abnormal transcript reads that mapped to neurobeachin-like protein 2 (NBEAL2). Genomic DNA sequencing confirmed the mRNA sequence abnormalities leading to the discovery of NBEAL-2 as the genetic cause of GPS (Kahr et al. 2011). Recent studies also revealed that patients with ETV6 (Ets Variant 6) germline mutations, who have thrombocytopenia and predisposition to lymphoblastic leukemia, display decreased expression of platelet-specific transcripts including significant reduction of mRNAs that regulate cytoskeletal processes (Noetzli et al. 2015). These studies suggest that ETV6, which is known to modulate the activity of other ETS transcription factors, is critical for transcriptional events that occur during platelet formation.

Platelet Transcripts to Monitor Response to Therapy

Aspirin treatment, or treatment with other antiplatelet agents, reduces the risk of primary cardiovascular events in hyperthrombotic states such as diabetes, chronic kidney

disease, coronary artery disease, or secondary cardiovascular events especially after stent placement (Hennekens and Dalen 2014; Park et al. 2015). Resistance to therapy, where platelets demonstrate high reactivity despite treatments, renders pharmacologic therapies less effective or ineffective (Floyd and Ferro 2014). mRNA studies in platelets and whole blood are being used to assess the mechanisms that drive resistance to therapies and as a potential method to screen for nonresponders.

Specific mRNAs differentially expressed in aspirin-resistant platelets have been determined (Fallahi et al. 2013). Regulators of G protein signaling (RGS), critical modulators of platelet function, were specifically analyzed for relationship to aspirin resistance in 39 aspirin-resistant and 50 aspirin-sensitive patients with metabolic syndrome (Mao et al. 2014). Transcripts for RGS2, RGS10, and RGS18 were significantly higher in the aspirin-resistant group. In another study, healthy volunteers were treated for 15 days with aspirin. MRp4 (an efflux transporter that can transport aspirin) mRNA and protein expression were increased by approximately 30 %. Human megakaryocytes grown in aspirin also increased mrp4 suggesting a direct effect of aspirin on platelet MRP4 expression (Massimi et al. 2014).

Another study measured whole blood mRNA levels in aspirin responders and nonresponders pre and post 2 weeks of aspirin treatment (Voora et al. 2013). A discovery cohort identified a gene set, called an aspirin responsive signature (ARS), that associated with aspirin responsiveness in the validation cohort as measured by a composite platelet function score. Although performed on whole blood, 24 of the 60 were platelet specific, and several strongly contributed to the correlation in the gene set, including the transcript ITGA2B. Interestingly, the ARS did not correlate with platelet function score in the absence of aspirin treatment. Furthermore, the ARS, or the expression of ITGA2B itself, was independently associated with the risk for acute MI and death.

The earlier studies demonstrate the promising approach for using mRNA to identify markers and drivers of aspirin or clopidogrel responsiveness. This approach could be used in other antiplatelet drug treatments, or even in therapies where platelets are not the primary targets, but may be involved in drug responsiveness such as the use of fibrinolytics or desmopressin. Considerations for how therapies might affect subsets of platelets, including young versus old platelets, will also be of interest. In this regard, dyes such as thiazole orange have been used to distinguish the immature platelet fraction that is thought to be more "mRNA rich" and individuals with a higher immature platelet fraction are less responsive to clopidogrel treatment (Ibrahim et al. 2012). Furthermore, RNA studies are not limited to mRNA. miRNAs are driving considerable interest in their field because of the ability of a single miRNA to target multiple mRNAs in a pathway. In this regard, miR-223 associates with high platelet reactivity in patients on clopidogrel (Shi et al. 2013).

Platelet Transcripts as Diagnostic Biomarkers of Disease

Although platelets contain some mitochondrial DNA, they lack nuclear DNA, and thus are generally refractory to acute (minutes to hours) transcript changes caused by sudden environmental alterations. Therefore, the total transcript pool in circulating platelets is thought to capture accumulated changes that occur in the ~1000–3000 platelets released from their parent megakaryocyte. As a result, systemic and chronic signals from infection, cancer, or inflammatory disease are more likely to shape the transcriptome of megakaryocytes and, as a consequence, be transferred into platelets. In addition, the accumulated uptake of exogenous RNA (Nilsson et al. 2011), either by direct cellular transfer, or paracrine and endocrine transfer via microparticles, may also alter the platelet transcriptome in disease situations (Clancy and Freedman 2014). Because platelets circulate systemically and are easily obtained, several recent studies have highlighted the potential use of platelet transcripts as biomarkers, even for diseases without apparent platelet etiology.

As an example, Wurdinger and colleagues. (Best et al. 2015) sampled platelets (called Tumor Educated Platelets or TEP) from 55 healthy individuals and 228 cancer patients spanning six different cancers. They included nonsmall cell lung carcinoma, colorectal cancer, glioblastoma, pancreatic, hepatobiliary, breast cancer, none of which are blood-related cancers. Comparing the cancer platelet transcriptome to healthy platelets, this group identified >1400 increased and ~800 decreased transcripts. Samples were subdivided into a training and validation cohort, and using a leave-one-out cross-validation support vector machine algorithm (SVM/LOOCV), 1072 RNAs, including coding mRNAs, were used to classify pan-cancer at a high sensitivity, specificity, and accuracy with high predictive strength. Using the same method, the authors found that platelet transcript signatures were able to accurately classify the origin of different subtypes of tumors, including in metastatic cancers, with high specificity and accuracy. Of note, some of the tumor-differentiating RNAs were transcripts not normally found in platelets (i.e., leukocyte-specific transcripts). It will be interesting to determine whether tumor-differentiating transcripts are from pathologically induced expression in megakaryocytes, transfer of mRNAs from leukocytes to platelets, or from an increased propensity of platelet–leukocyte aggregates in cell isolations collected from cancer patients.

Regardless of the source of the differences in RNA expression, and independent of the impact of these changes to platelet function, the application of a platelet signature is a promising new avenue for a liquid biopsy-based cancer diagnostic and classification.

Another promising diagnostic avenue might be the direct detection of cancer causing mutations in platelets. In the Wurdinger study (Best et al. 2015), mutant KRAS RNA, a mutation common to cancers, was detected in some platelet samples. In another study by the same group (Nilsson et al. 2016), RT-PCR detected EML4-ALK rearrangements in platelet RNA from individuals with nonsmall cell lung cancers. Rearrangements were detected with 65 % sensitivity and 100 % specificity as compared to matched tissue biopsies. Of 77 cancer patients, rearrangements were detected in platelets from 22 patients. The tumor RNA was enriched in platelets compared to plasma, where the rearrangement was only detected in three patients. Because EML4–ALK fusion in cancers is related to progression-free survival, RT-PCR in platelets could be used to serially monitor for EML4–ALK presence and the probability of progression-free survival (Nilsson et al. 2016).

The potential use of platelet transcripts in disease diagnosis and tracking has also expanded to neurodegenerative disorders. Platelets contain many transcripts that are enriched in brain cells, including amyloid precursor protein (APP) (Catricala et al. 2012). RT-PCR analysis of APP mRNA in platelets from Alzheimer's disease (AD) or Frontotemporal Lobar Degeneration (FTLD), indicated upregulation of this mRNA in both diseases, and the level correlated with cognitive impairment in the AD group (Vignini et al. 2013). These studies in cancer and neurodegenerative disorders highlight the possible utility of platelet mRNA as a diagnostic biomarker of disease.

mRNA Profiles in Mouse Platelets

The primary role of platelets to prevent excess bleeding and promote wound healing is shared between human and mouse, and mouse models are often used to understand human platelet function. Understanding similarities and differences between humans and mice is critical for accurate interpretation of murine models of platelet function. For example, thrombin activates human platelets through Par1, but alternatively uses Par3 on mouse platelets (Ishihara et al. 1997; Kahn et al. 1998). Humans respond robustly to platelet activating factor, whereas mouse platelets are not responsive (Karpouza and Vakirtzi-Lemonias 1997). As one might predict, initial RNA-seq comparisons between human and mouse transcriptomes have identified similarities and differences in platelet mRNA expression (Rowley et al. 2011). While the majority of transcripts are expressed at similar levels, several show extreme differences in expression including mRNAs for Par1, Par3, and platelet-activating factor receptor. Thus, mouse models can now be interpreted in the light of molecular expression differences revealed through RNA-seq. Moreover, mRNA profiling can also be used to identify secondary mRNA changes induced by specific mouse genetic knockouts to guide the phenotyping of these animals.

Summary

Characterizing mRNA expression profiles in circulating platelets has emerged as a powerful tool for understanding processes in megakaryocytes and their offspring. It is becoming more appreciated that the transcriptome of platelets is complex and diverse (Fig. 3), and the repertoire of mRNAs expressed by platelets changes in disease. These changes likely reflect differential shuttling of mRNAs from megakaryocytes to proplatelets in pathologic situations, but may also result from horizontal mRNA transfer from other circulating cells.

Determining mRNA expression patterns in platelets is informative on several fronts including: (1) assessment of translation; (2) providing insight into thrombopoiesis; (3) understanding the molecular phenotype and function of platelets from people of different races, gender, and age as well as patients with underlying genetic disorders or acquired diseases; (4) evaluating platelets during storage and their efficacy for transfusion; and (5) predicting the expression of proteins and conservation of genes between mouse and human platelets, which is critical for investigators pondering whether to knock-out or knock-in genes in mice for in vivo appraisal of platelet function. Although powerful, global transcriptome profiling is time consuming, costly, and comes with the difficult task of bioinformatically dissecting the data and determining the next steps of experimental pursuit. The good news is that significant information regarding the platelet transcriptome can be obtained by simply accessing published NGS datasets, and specific genes can be assessed at the candidate level for minimal costs. More sophisticated bioinformatics analyses and advanced NGS-based techniques such as ribosomal footprinting will undoubtedly emerge, and results from these studies will provide new information regarding the megakaryocyte and platelet transcriptome and how mRNA is linked to platelet function in health and disease.

Take Home Messages

- Next-generation transcriptome wide sequencing (RNA-seq) has vastly expanded our current understanding of the platelet transcriptome.

(continued)

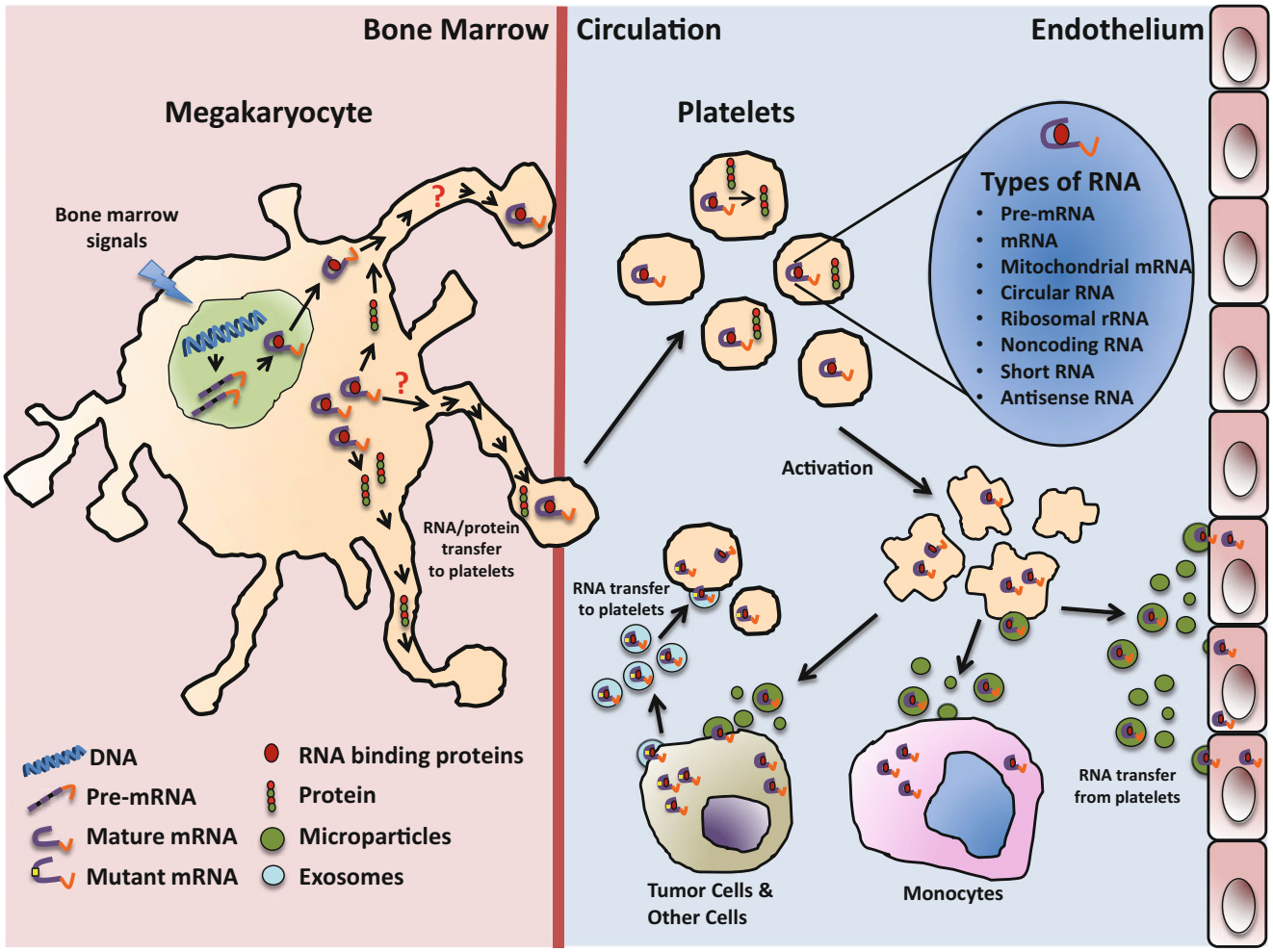

Fig. 3 The fate of platelet and megakaryocyte mRNA. Pre-mRNAs are transcribed in the megakaryocyte where the majority are processed into mature mRNA and exported from the nucleus for translation. Megakaryocyte mRNAs and their protein products are transferred, sometimes differentially, into proplatelets and their platelet progeny, but the mechanisms of transfer are not clear. Depending on the environmental and genetic signals influencing megakaryocyte RNA transcription and turnover at the time of platelet production, different RNAs may be ultimately transported into platelets. Constitutive or signal-dependent translation of mRNA can occur in circulating platelets. Once platelets are released into the circulation their mRNA content (and thereby potential protein production and function) is altered by degradation, by uptake of additional mRNA from other cells, including from tumor cells, or transport of their mRNA to other cells

- The platelet transcriptome is complex, containing all the major RNA subtypes.
- The source of platelet mRNAs is not clear, but most are probably directly captured from megakaryocytes, while others may be transferred into platelets.
- Platelet mRNAs are stable and their mRNA profiles are snapshots of the cumulative transcriptional events that occurred in megakaryocytes.
- Platelet mRNAs code for corresponding proteins, and platelet mRNAs can be used to predict protein and functional differences in health and disease.

- Platelet mRNAs are being used to understand genetic differences in platelet function, including race, gender, and platelet disorders.
- Platelet mRNAs may be used to monitor response to therapy such as aspirin responsiveness.
- Platelet mRNAs may be used as biomarkers of a variety of diseases including metabolic syndromes, infectious diseases, and cancer.
- Mouse platelet mRNA profiles can guide the design and characterization of platelet-related mouse models.

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Noncoding RNAs in Platelet Biology

Leonard C. Edelstein and Paul F. Bray

Abstract

Noncoding RNAs (ncRNA) account for the majority of the human cellular transcriptome. Advances in sequencing technologies and analytic approaches continue to identify novel ncRNAs. A large amount of research has investigated the role of microRNAs (miRNAs) in megakaryopoiesis and platelet biology, whereas relatively little is known about other ncRNAs. miRNAs affect basic development and physiology by regulating protein translation, and in doing so miRNAs regulate hematopoiesis and megakaryopoiesis. To date, platelet miRNA studies have focused on their use as biomarkers and as tools for understanding basic mechanisms of megakaryocyte/platelet gene expression, but there is a rapidly expanding appreciation for miRNAs as mediators or modifiers of disease, the potential for platelet miRNAs to affect gene expression in other tissues, and as therapeutic targets.

Introduction

The importance of noncoding RNA (ncRNA) in cell biology has been appreciated since the discovery of transfer RNAs (tRNA) and ribosomal RNAs (rRNAs) and their essential role in protein synthesis. However, the emphasis on small ncRNAs is relatively recent, beginning with the discovery of microRNAs (miRNAs) in the early 1990s. The introduction of next-generation RNA sequencing (RNA-seq) together with sophisticated bioinformatic analyses of large amounts of sequence data without a priori sequence information has led to the discovery of numerous other classes of ncRNAs. In addition to protein synthesis, many aspects of RNA biosynthesis are regulated by ncRNAs including splicing by small nuclear RNAs (snRNAs), rRNA processing by small nucleolar RNAs (snoRNAs), and mRNA translation and stability by microRNAs (miRNAs). ncRNA categories now include PIWI-associated RNAs (piRNAs) that regulate chromatin modification, and small Cajal body-associated RNA (scaRNAs) that modify snRNAs (Cech and Steitz 2014). A common theme among many of these categories is RNA-mediated regulation of RNA processes. This chapter will discuss the likely influence of ncRNAs on megakaryocyte (MK) gene expression and value of understanding ncRNAs in peripheral blood platelets.

miRNAs

MiRNAs are approximately 22-nucleotide regulatory RNAs expressed in multicellular organisms (Bartel 2004). miRBase v21 (June 2014, http://www.mirbase.org/) lists 2588 mature human miRNAs while the GENCODE reference set (v22) derived from ENCODE data lists 4093 (Derrien et al. 2012), although recent data provides strong evidence for more than twice that many (Londin et al. 2015). miRNAs regulate most (>60 %) mammalian protein-coding genes (Friedman et al. 2009). Some miRNAs are expressed ubiquitously, but many are tissue and/or developmental stage specific (Wienholds et al. 2005; Londin et al. 2015).

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MiRNA biogenesis has been reviewed in depth elsewhere (Krol et al. 2010) but will be briefly summarized here (Fig. 1). The canonical miRNA pathway consists of pri-miRNAs transcribed from DNA genes by RNA polymerase II, which are then processed into pre-miRNAs in the nucleus by a Drosha RNase-containing complex. After exportation to the cytoplasm Dicer shortens the pre-miRNA into a ~22 nucleotide double-stranded RNA molecule. One strand of this molecule is loaded into the RNA-induced silencing complex (RISC) that contains Argonaute proteins. Guided by the miRNA sequence, the RISC then causes translational inhibition followed by mRNA degradation (Guo et al. 2010; Bazzini et al. 2012; Djuranovic et al. 2012). The impact of miRNAs on gene expression is often to fine-tune and reduce noise in protein expression (Selbach et al. 2008; Baek et al. 2008; Schmiedel et al. 2015). In some cases, as little as a 20 % change in miRNA levels has been shown to produce disease or predispose to malignancy (Alimonti et al. 2010).

miRNAs target multiple mRNAs, and most mRNAs are targeted by multiple miRNAs. mRNA target prediction is a critical aspect of miRNA research, because it is through the targets that miRNA function is achieved. Numerous different miRNA target prediction algorithms are publicly available, including TargetScan, Miranda, PicTar, RNA22, and Microcosm. However, there is a lack of consensus on the optimal prediction method (Rigoutsos and Tsirigos 2011). Often, researchers utilize multiple algorithms that assign different "weights" to various features, such as degree of sequence homology, species conservation, etc., although even this approach has not been validated. Because of false-positive target predictions, it is essential to test in silico predictions in cells.

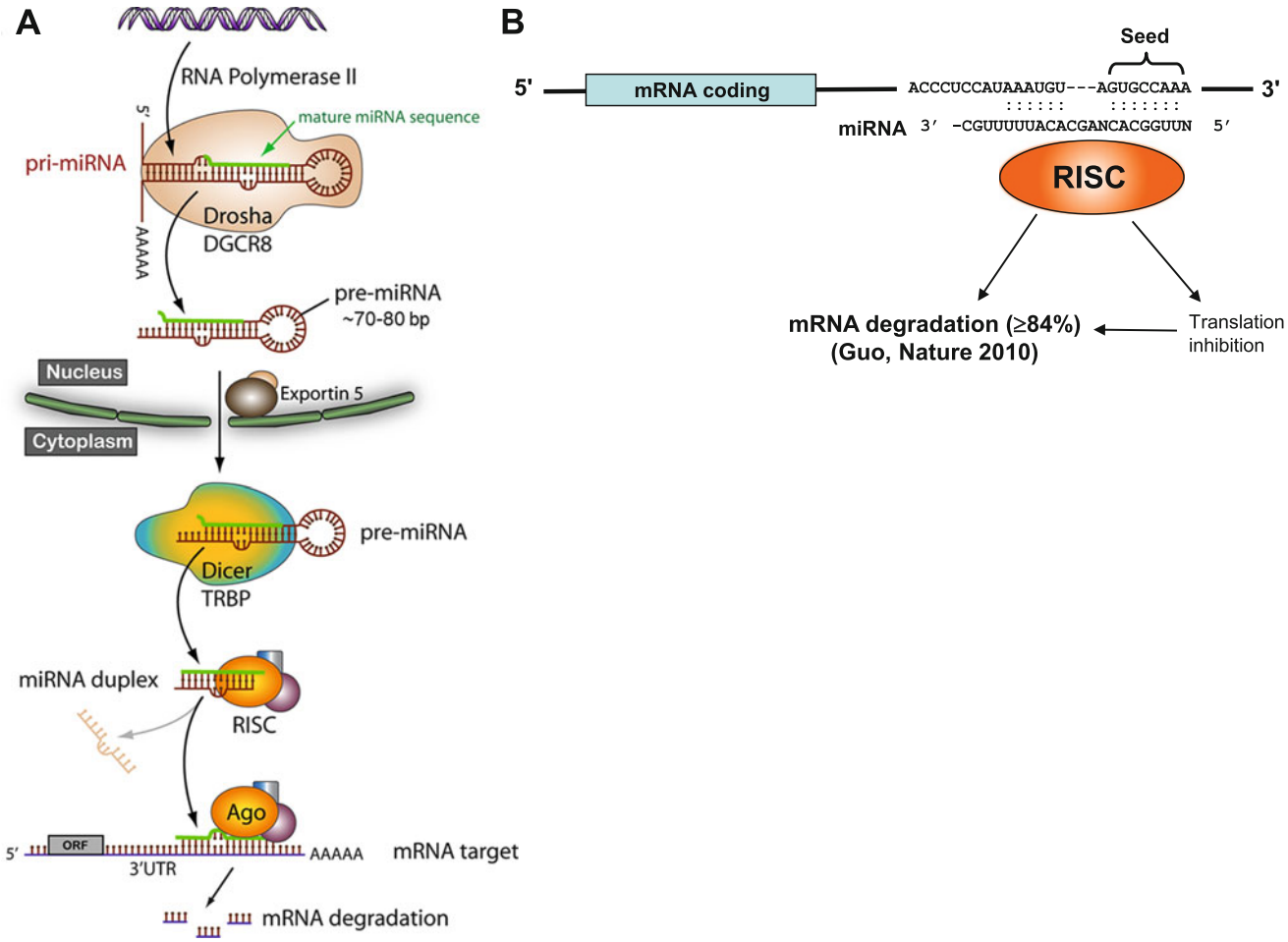

Fig. 1 MicroRNA biogenesis and function. (**a**) The canonical miRNA biosynthesis pathway, described in text. *Drosha* an RNase type III endonuclease, *DGCR8* DiGeorge syndrome critical region 8, *Dicer* another RNase type III endonuclease, *TRBP* TAR RNA-binding protein, *RISC* RNA-induced silencing complex, *Ago* Argonaute 2, *ORF* open reading frame. (**b**) The seed region of the miRNA (bp 2-8) bind to complementary sites on the 3′ UTR of target mRNAs where it predominantly causes a decrease in mRNA levels, but may also inhibit translation. Figure from Edelstein and Bray (2011)

miRNAs and Megakaryopoiesis

The important role of miRNAs in both healthy and malignant hematopoiesis has been thoroughly investigated (Lazare et al. 2014). The function of miRNAs has been studied using a number of techniques including profiling human and mouse $CD34^+$ hematopoietic stem cells differentiated in culture, and genetically manipulating mice and human $CD34^+$ cells, human embryonic stem cells, and transformed cell lines with megakaryocytic properties. These approaches have identified numerous miRNAs that positively and negatively influence megakaryocytic differentiation. Figure 2 summarizes the miRNAs regulating megakaryopoiesis that are described later.

miRNAs Positively Regulating Megakaryopoiesis

miR-150 was one of the first miRNAs identified as regulating megakaryopoiesis (Lu et al. 2008). Different lineages of primary cord blood hematopoietic cells at various stages of differentiation were isolated by flow cytometry and miRNA profiles were determined. *miR-150* was enriched in megakaryocytes as compared to megakaryocyte-erythroid precursors (MEPs). Forced expression in murine bone marrow cells resulted in an increase in colony forming units-megakaryocytes (CFU-MKs). Subsequently, *miR-150,* whose expression increases in response to thrombopoietin (TPO), targets and downregulates *MYB*, a transcription factor that is a negative regulator of hematopoiesis, offering a model of how *miR-150* affects MK development (Emambokus et al. 2003; Barroga et al. 2008).

miR-34a also enhances megakaryopoiesis. *MYB*, as well as *CDK4* and *CDK6,* is targeted by *miR-34a*, a miRNA whose expression level increases during phorbol ester-induced differentiation of K562 cells and TPO-induced differentiation of $CD34^+$ cells. Enforced expression of *miR-34a* in K562 cells enhanced MK differentiation, as measured by cell surface markers (Navarro et al. 2009; Ichimura et al. 2010).

miR-146b increases in phorbol-ester-treated K562 cells and ectopic expression of *miR-146b* in these cells enhanced MK development (Zhai et al. 2014). Platelet-derived growth factor receptor α (*PDGRA*), a negative regulator of megakaryopoiesis, was identified as a target of *miR-146b*.

miR-105 was identified as a positive regulator of megakaryopoiesis in a high-throughput screen in which miRNAs were overexpressed in human embryonic stem cells driven to differentiate into multiple lineages (Kamat et al. 2014).

The earlier megakaryopoiesis-regulating miRNAs were identified using unbiased approaches. In contrast, Chapnik et al. generated a *miR-142* null mouse, specifically because of its known role in hematopoiesis. The mice exhibited reduced MK maturation, proplatelet formation, and thrombocytopenia (Chapnik et al. 2014), supporting *miR-142* as a positive regulator of megakaryopoiesis.

miRNA Negatively Regulating Megakaryopoiesis

By silencing *MYB* in human $CD34^+$ cells, Bianchi and colleagues identified *miR-486-3p* as a target of *MYB*-induced transcription (Bianchi et al. 2015). They further identified

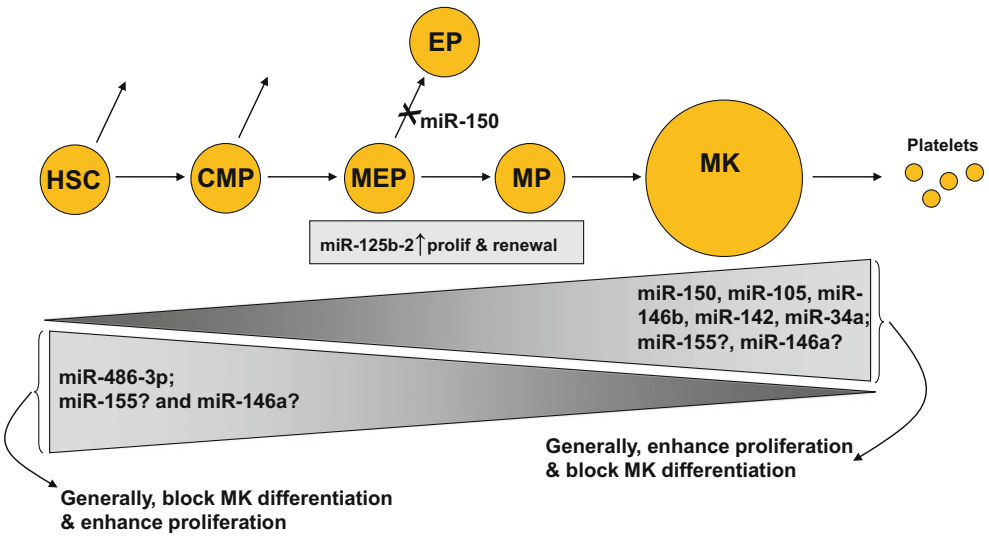

Fig. 2 MicroRNAs and megakaryocytopoiesis. This cartoon summarizes the miRNAs known to participate in megakaryocytopoiesis. Megakaryocytes (MK) originate from self-renewing hematopoietic stem cells (HSC), which differentiate progressively into common myeloid precursors (CMP), megakaryocyte/erythroid precursors (MEP), and MK precursors (MP). The height of the triangles indicates the level of the miRNA, with some miRNAs decreasing during megakaryocytopoiesis, and others increasing. There is conflicting evidence regarding the role of miR-146a and miR-155. Levels of miR-125b-2 do not change during progression from MEP to MP. Figure updated from Edelstein and Bray (2011)

MAF, another transcription factor, as a target of *miR-486-3p*-mediated negative regulation. Overexpression of *miR-486-3p* or silencing *MAF* expression in human $CD34^+$ cells inhibited MK differentiation. Taken together, these studies support an axis in which *miR-150* and *miR-34a* (positive regulators of megakaryopoiesis) target and reduce *MYB* levels resulting in lower levels of its transcriptional target, *miR-486-3p*. Lower levels of *miR-486-3p* result in higher levels of *MAF*, and thus, enhanced megakaryopoiesis.

miRNAs with Complex Effects on Megakaryopoiesis

miR-155 was identified as a hematopoietic stem cell miRNA predicted to target mRNAs critical to hematopoiesis. When transduced into human $CD34^+$ cells, *miR-155* resulted in decreased numbers of CFU-MKs (Georgantas et al. 2007). Identified targets of *miR-155* include the *Ets1* and *Meis1* transcription factors, well known regulators of megakaryocytopoiesis, as well as the chromatin remodeling protein *JARID2* (Romania et al. 2008; Norfo et al. 2014). In contrast to the data indicating that increased *miR-155* decreases megakaryopoiesis, silencing of *JARID2* in human $CD34^+$ cells resulted in increased numbers of MK precursors (Norfo et al. 2014). This data, along with work demonstrating that transfer of hematopoietic stem cells with enforced expression of *miR-155* results in decreased bone marrow MKs, erythrocytes, and lymphocytes but a large number of splenic MKs, suggests that *miR-155* plays a critical role in MK development in a complex manner that depends on the developmental stage (O'Connell et al. 2008).

miR-146a was identified as a transcriptional target negatively regulated by the promyelocytic leukemia zinc-finger (PLZF), a transcription factor known to be involved in hematopoiesis and leukemogenesis. Labbaye et al. reported that *miR-146a* expression decreased in human $CD34^+$ cells induced to differentiate into MKs and that ectopic expression of *miR-146a* in human $CD34^+$ cells inhibited megakaryocytic differentiation, as measured by reduced number of cells with polylobulated nuclei, surface markers, and CFU-MKs (Labbaye et al. 2008). In contrast, Opalinska et al. found that *miR-146a* increased during differentiation and had no effect on megakaryopoiesis (Opalinska et al. 2010). Starczynowski et al. (2010, 2011) reported that the level of *miR-146a* is lower in MK/erythroid precursors in mice relative to hematopoietic stem cells, similar to what Labbaye et al. (2008) found in human cells, but that forced expression in mice had no effect on platelet number, similar to observations by Opalinska et al. (Opalinska et al. 2010; Starczynowski et al. 2011). However, when *miR-146a* was downregulated in mice by decoy targets or anti-miRNA locked nucleic acids (LNA), the CFU-Mk and platelet number increased and the ploidy of the MKs decreased, similar to Labbaye et al. (Starczynowski et al. 2010; Labbaye et al. 2008). Taken together, there is strong evidence that *miR-146a* modifies megakaryopoiesis, but the effects are complex and may differ under different conditions or stresses that have yet to be defined.

The Platelet miRNA Repertoire

Because platelets are more accessible than MKs, more data characterizing the platelet transcriptome has been obtained. Platelets from healthy individuals contain approximately 6000 different protein-coding transcripts (Simon et al. 2014; Londin et al. 2014; Bray et al. 2013; Gnatenko et al. 2003; Rowley et al. 2011) and at least 178 commonly expressed miRNAs (Edelstein et al. 2013). Table 1 lists the highest expressed miRNAs in platelets from healthy subjects (Edelstein et al. 2013; Simon et al. 2014). Many reports over the past several years suggest platelet miRNAs are biologically and clinically relevant as (1) tools to understand platelet physiology and MK gene expression, (2) potential regulators of platelet RNA and protein expression levels, and (3) a source for circulating miRNAs.

Recent work has identified novel platelet-specific miRNAs previously unknown because reference databases did not include RNA-seq of platelet RNA (Londin et al. 2014, 2015). In addition to the 2588 miRNAs listed in miRBase v21, Londin et al. analyzed RNA-Seq data from 1323 samples from 13 different human tissues, including ten platelet samples. They were able to identify 3707 novel miRNAs derived from 3494 precursors. Sixty-eight of these novel miRNAs were detected in all ten platelet samples including 11 which were platelet specific (Londin et al. 2015). Given the large number of predicted targets for each miRNA, these miRNAs may have a significant impact on megakaryocyte/platelet regulation of the transcriptome.

miRNAs in Platelet Physiology and Gene Expression

Because miRNAs often target hundreds of mRNAs, miRNA function is not obvious. Association studies between miRNA levels and cell parameters are a useful approach for beginning to understand potential functional effects of miRNAs. The well-established interindividual variation in platelet number and function, plus the ease of obtaining platelets, makes platelets an ideal system for studying miRNA function in general, and for platelet miRNAs specifically. Although RNA profiling can identify novel regulators of platelet function in healthy and disease conditions, some

Table 1 Forty highest platelet-expressed miRNAs

Rank	Symbol	Mirbase accession	Chromosome
1	hsa-mir-223	MIMAT0000280	chrX
2	hsa-mir-26a	MIMAT0000082	chr12
3	hsa-mir-126	MIMAT0000445	chr9
4	hsa-mir-142-3p	MIMAT0000434	chr17
5	hsa-mir-16	MIMAT0000069	chr13
6	hsa-mir-92a	MIMAT0000092	chr13
7	hsa-mir-21	MIMAT0000076	chr17
8	hsa-mir-103	MIMAT0000101	chr20
9	hsa-mir-20a+hsa-mir-20b[a]	NA	NA
10	hsa-let-7a	MIMAT0000062	chr11
11	hsa-mir-24	MIMAT0000080	chr19
12	hsa-let-7g	MIMAT0000414	chr3
13	hsa-mir-199a-3p+hsa-mir-199b-3p[a]	NA	NA
14	hsa-mir-15a	MIMAT0000068	chr13
15	hsa-let-7d	MIMAT0000065	chr9
16	hsa-mir-30b	MIMAT0000420	chr8
17	hsa-let-7f	MIMAT0000067	chr9
18	hsa-let-7i	MIMAT0000415	chr12
19	hsa-mir-15b	MIMAT0000417	chr3
20	hsa-mir-23a	MIMAT0000078	chr19
21	hsa-mir-221	MIMAT0000278	chrX
22	hsa-mir-146a	MIMAT0000449	chr5
23	hsa-mir-451	MIMAT0001631	chr17
24	hsa-mir-19b	MIMAT0000074	chr13
25	hsa-mir-142-5p	MIMAT0000433	chr17
26	hsa-mir-148b	MIMAT0000759	chr12
27	hsa-mir-191	MIMAT0000440	chr3
28	hsa-mir-25	MIMAT0000081	chr7
29	hsa-mir-106b	MIMAT0000680	chr7
30	hsa-mir-423-5p	MIMAT0004748	chr17
31	hsa-mir-27b	MIMAT0000419	chr9
32	hsa-mir-340	MIMAT0004692	chr5
33	hsa-mir-181a	MIMAT0000256	chr1
34	hsa-mir-151-5p	MIMAT0004697	chr8
35	hsa-mir-720	MIMAT0005954	NA
36	hsa-mir-199a-5p	MIMAT0000231	chr1
37	hsa-mir-101	MIMAT0000099	chr1
38	hsa-mir-374b	MIMAT0004955	chrX
39	hsa-mir-29a	MIMAT0000086	chr7
40	hsa-mir-148a	MIMAT0000243	chr7

[a]Some probes were nonunique and detected multiple species of miRNA. These probes are indicated by listing the detected species in a single row

caution is needed when interpreting the results: (1) Leukocytes contain 60–200 times as much miRNA as platelets (Teruel-Montoya et al. 2014), so proper platelet purification is necessary. Immunodepletion of leukocytes and erythrocytes can result in greater than $1{:}10^6$ leukocyte: platelet ratio, allowing for a more accurate representation of the platelet transcriptome. (2) Because many platelet miRNA levels differ by subject age, race, gender, and medication use (Edelstein et al. 2013; Simon et al. 2014), these variables must be considered in any analyses comparing different groups of subjects. (3) Caution must also be used when assessing platelet miRNA levels and function in transformed cell lines since miRNA expression data has shown that cell line miRNA profiles more closely resemble each other than their postulated cell of origin (Teruel-Montoya et al. 2014).

Kondkar et al. first reported that compared to hyporeactive platelets, *VAMP8* mRNA is more highly expressed in hyperreactive platelets (Kondkar et al. 2010). These investigators showed that *miR-96* levels were inversely correlated with

both *VAMP8* mRNA levels and platelet hyperreactivity, and that *miR-96* regulated *VAMP8* mRNA and protein levels. Subsequently, it was shown that miRNA expression profiles predicted platelet aggregation response (Nagalla et al. 2011). Using a bioinformatics approach, three miRNA:mRNA pairs—*miR-200b:PRKAR2B, miR-495:KLHL5, miR-107:CLOCK*—were identified and validated experimentally. This was the first demonstration of the functional importance of the regulatory subunit of PKA in human platelets.

The Platelet RNA and eXpression 1 (PRAX1) study performed detailed platelet phenotyping, genome-wide genotyping, genome-wide mRNA and miRNA profiling, and tested for multi-omic associations (Edelstein et al. 2013). Platelets from black subjects were discovered to be more responsive to activation through the protease-activated receptor 4 (PAR4) thrombin receptor. A large number of miRNAs and mRNAs were differentially expressed by both self-identified race and PAR4 reactivity (Edelstein et al. 2013). Among the miRNAs differentially expressed by race was the *DLK1-DIO3* cluster of 54 miRNAs. One member of this cluster, *miR-376c*, was shown to regulate phosphatidylcholine transfer protein (*PCTP*), which itself is differentially expressed by race. PC-TP protein was then confirmed as a regulator of platelet PAR4 function. These data are consistent with the possibility that differentially expressed miRNAs contribute to the greater thrombotic risk in blacks as compared to whites (Mozaffarian et al. 2015; Thomas et al. 2010).

Despite the absence of a nucleus, platelets contain mRNA, splice mRNAs, and translate mRNA into proteins relevant to hemostasis and inflammation (Warshaw et al. 1966; Weyrich et al. 2009; Denis et al. 2005). Human platelets also contain functional miRNA processing machinery and miRNAs that are complexed with Ago2 (Landry et al. 2009). There is an increasing body of evidence indicating that inflammatory states enhance platelet mRNA translation into protein (Hottz et al. 2013). Supporting this finding, Corduan et al. reported that upon platelet activation, mRNAs are released from the Ago2:miRNA complex and undergo translation (Corduan et al. 2015). Finally, platelets continue to translate proteins from mRNA under Blood Bank storage conditions (Thon and Devine 2007).

Platelet miRNAs in Disease

Altered miRNA expression has been associated with numerous clinical conditions. Such associations may have value as diagnostic biomarkers or to better understand the molecular pathophysiology of the disease. Because many of these studies utilize blood as the RNA source for profiling, it is important to know which cellular or noncellular component of the blood was used. Extrapolating results from plasma RNA to the cell of origin is only appropriate insofar as the profiles of the individual components are known and if the results are "normalized" to the amount of total RNA assayed. Recently, a formal approach was utilized to determine the absolute quantity of RNA per blood cell (Teruel-Montoya et al. 2014). Although nucleated blood cells have the highest miRNA content per cell (Table 2), because of cell number differences erythrocytes contribute more cellular miRNA to the blood, followed by granulocytes and platelets. Not surprisingly, miRNA expression patterns differ for each lineage (Fig. 3). These lineage differences are important to consider when interpreting disease-circulating miRNA association studies.

Hematologic Disease Starczynowski and colleagues showed that patients with myelodysplasia and deletions of 5q lost *miR-145* and *miR-146a*. They further showed that knockdown of these miRNAs in mouse hematopoietic stems cells caused thrombocytosis, neutropenia, and megakaryocytic dysplasia in a manner dependent upon *miR-145* and *miR-146a* inhibition of tumor necrosis factor receptor-associated factor-6 (Starczynowski et al. 2010). Whereas most other studies have identified disease *associations*, the Starczynowski study is one of the few studies demonstrating a *causal* role for platelet miRNAs in disease pathophysiology.

Polycythemia rubra vera (PV) and essential thrombocytosis (ET) are BCR-ABL negative myeloproliferative neoplasms that are difficult to distinguish from nonclonal conditions due to the variety of causative mutations. A study with PV patients demonstrated higher levels of *miR-26b* in the platelets of patients with PV compared to control subjects (Bruchova et al. 2008). Subsequent work showed that as few as three miRNAs (*miR-10a, miR-148a*, and *miR-490-5p*)

Table 2 Contribution of each cell type to total miRNA content per volume of blood

	Platelet	T-Cell	B-Cell	Granulocyte	Erythrocyte
Total RNA mass, femtogram/cell	2.20	2188.00	1360.00	646.30	0.57
miRNA mass, femtogram/cell	0.05	3.28	3.16	10.23	0.04
miRNA mass, picogram/μl blood	16.90	3.55	0.64	50.24	184.00

Five cell types from five donors were purified and total RNA was extracted. Based on original cell counts, the average total RNA yield on a per cell basis was calculated (row 1). RNA samples were size profiled, the average miRNA-to-total RNA ratio was calculated for each cell type from all subjects, and based on the average total RNA per cell, the average miRNA mass per cell type was calculated (row 2). Row 3 has estimates of the contribution of each hematopoietic cell type to the miRNA content of blood volume using cell miRNA content and cell number per blood volume. Table from Teruel-Montoya et al. (2014)

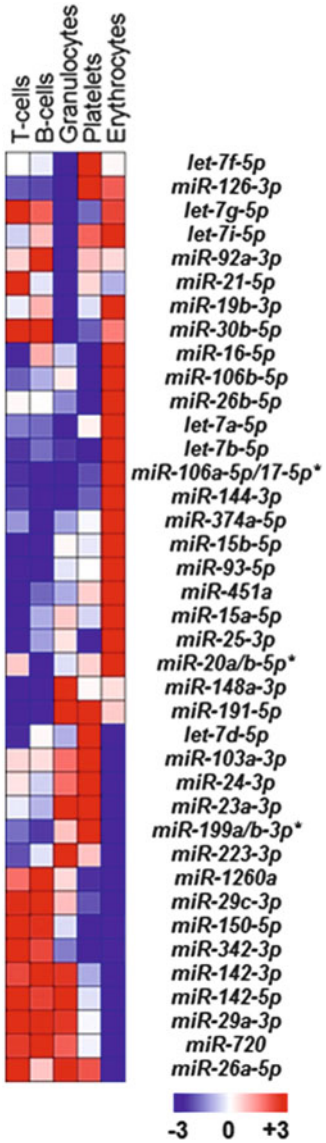

Fig. 3 Peripheral blood cells miRNA profiles. Heatmap is derived from the union of the most abundant miRNAs (39 in total) in each primary blood cell type. Each column indicates the average log-ratio intensity data. *Asterisk* indicates probes with similar and indistinguishable sequence with the nCounter platform. Figure from Teruel-Montoya et al. (2014)

could discriminate ET from normal platelets (Xu et al. 2012). Platelets from a small number of patients with sickle cell anemia were reported to have different platelet miRNA expression profiles than platelets from nonsickle disease subjects (Jain et al. 2013). Unfortunately, none of these clinical association studies considered demographic variables known to alter miRNA expression levels.

Cardiovascular Disease Platelets play a central role in the pathophysiology of acute and chronic cardiovascular disease. Blood cell miRNA profiles were observed to differ between patients having ST-segment elevation MI or non-ST-segment elevation MI with platelet *miR-25-3p* and *miR-221-3p* significantly higher in patients with STEMI than NSTEMI (Ward et al. 2013). A small study of 12 patients with premature CAD and 12 healthy controls identified differentially expressed platelet miRNAs (Sondermeijer et al. 2011). Notably, the majority of studies testing for associations between miRNAs and cardiovascular disease utilize plasma or cardiac tissue, but not purified platelets as an RNA source, and confounding demographic variables altering miRNA levels were not considered. Of potential interest is a report by Zampetaki et al. who prospectively tested for associations between circulating miRNAs and incident MI (Zampetaki et al. 2012). These investigators found *miR-126* levels were positively associated with incident MI, while *miR-223* levels were negatively associated with incident MI. Among circulating blood cells these are the two highest expressed platelet miRNAs, with the former being predominantly of platelet origin (Teruel-Montoya et al. 2014). However, this analysis is confounded by the known high levels of *miR-126* and *miR-223* in endothelial cells and granulocytes, respectively.

Platelet miR-223 in Disease

Generally recognized as the most abundant miRNA in platelets, *miR-223* has been investigated by many groups for its role in disease. While Stratz et al. reported that there was no overall change in the platelet miRNA profile of patients with type 2 diabetes, Elgheznawy and colleagues have reported that *miR-223* is lower in diabetic subjects (Elgheznawy et al. 2015; Stratz et al. 2014). This discrepancy may be due to differences in the presence of antiplatelet medication or experimental approach (profiling vs candidate gene). Elgheznawy et al. also reported that in diabetic platelets, calpain cleaves Dicer, resulting in lower levels of *miR-223, miR-142, miR-143,* and *miR-155*. Coagulation factor XIII-A was identified using proteomic and molecular techniques as a *miR-223* target. Mice deficient in *miR-223* exhibited shorter bleeding times, larger $FeCl_3$-induced thrombi, greater sensitivity to low doses of thrombin, and impaired clot retraction (Elgheznawy et al. 2015). These results differed from Leierseder et al. who reported that *miR-223* deficient mice had no defects in platelet number or function (Leierseder et al. 2013). However, these dissimilarities may be due to differences in assay type and agonist concentration. Platelet *miR-223* expression has also been reported to be lower in subjects with increased on-clopidogrel activity (Shi et al. 2013). And compared to platelets from healthy subjects, platelets from nonsmall cell

lung cancer patients have ten times higher levels of *miR-223* (Liang et al. 2015).

miRNAs in Stored Platelets

Platelets undergo a gradual loss in quality when stored for days at either room temperature or refrigerated (Shrivastava 2009). Attempts to limit bacterial contamination in room temperature-stored platelets include the use of nucleic acid crosslinking agents. Initially, Osman et al. studied the effect of these pathogen reduction systems on miRNA content and found that one of them, INTERCEPT, reduced the levels of 6 of the 11 tested miRNAs. However, neither miRNA crosslinking, nor alterations in synthesis or function was observed (Osman et al. 2015b). In a follow-up study utilizing RNA-Seq, Osman observed 147–800 mRNAs altered by the INTERCEPT system depending on stringency cutoffs (Osman et al. 2015a). Yu et al. measured miRNA levels in platelets 1, 3, and 5 days after apheresis and found ten miRNAs with altered expression levels (Yu et al. 2014). Of these, *miR-326* increased over time and was found to downregulate expression of the antiapoptotic *Bcl-xL*, resulting in apoptosis of the platelets (Yu et al. 2015). Deep sequencing of platelets stored for 1–5 days revealed a gradual loss of the most abundant miRNAs and suggests miRNA profiles might have utility as biomarkers for stored platelet quality (Cox et al. 2015).

miRNA Transfer by Platelet Microparticles

Microparticles are small (0.1–1 μm) vesicles shed by many cell types including platelets upon activation (Italiano et al. 2010). In addition to their protein contents, microparticles also contain miRNAs (Hunter et al. 2008). Notably, microparticles and platelet-like particles are able to transfer miRNAs to other cells types (Hunter et al. 2008; Yuan et al. 2009; Risitano et al. 2012; Gidlöf et al. 2013; Laffont et al. 2013; Pan et al. 2013), raising the possibility that miRNAs released upon platelet activation could regulate gene expression in other cells, such as endothelial, monocytic, neutrophilic, or smooth muscle cells. Platelet microparticle (PMP) engulfment and miRNA delivery to neutrophils have recently been reported to depend on the presence of platelet 12-lipoxygenase and secreted phospholipase A_2-IIA (Duchez et al. 2015).

PMPs have been shown to transfer miRNA to endothelial cells in vitro (Laffont et al. 2013). In addition, PMPs contain functional Ago2:*miR-223* complexes that can regulate *FBXW7* and *EFNA1* expression in endothelial cells. Consistent with the hypothesis that the PMP miRNA profile reflects the parental platelet, thrombopoietin was reported to increase *miR-223* maturation in platelets, leading to higher levels of *miR-223* in PMPs (Pan et al. 2013). This increase in *miR-223* led to decreased *IGF1-R* expression in cultured endothelial cells resulting in apoptosis. After demonstrating that a majority of circulating cell-free *miR-223* is contained in microparticles, Shan et al. injected a locked nucleic acid *miR-223* inhibitor into an atherosclerotic mouse model. This resulted in increased aortic lesion size and increased intimal: medial ratio in a model of carotid artery ligation injury, suggesting PMP *miR-223* has antiatherogenic properties (Shan et al. 2015). Platelet *miR-223* can be delivered to A549 lung cancer cell line where it targets and knocks down *EPB41L3* and increases the invasion capacity in an in vitro assay (Liang et al. 2015).

When Gidlöf and colleagues profiled the miRNA content of platelets from STEMI patients, they found *miR-22, miR-185, miR-320b,* and *miR-425-5p* were reduced in the platelets of patients compared to controls. These miRNAs were even lower in the thrombus as compared to circulating platelets, suggesting platelet activation had led to miRNA release. They further demonstrated that PMP delivered *miR-320b* could repress *ICAM-1* expression in cultured endothelial cells (Gidlöf et al. 2013). Finally, PMPs have been reported as delivering 34 miRNAs to human macrophages including *miR-126-3p*. This results in the downregulation of 367 mRNAs, including the cytokines *CCL4*, *CSF1*, and *TNF* (Laffont et al. 2015). Taken together the evidence for platelet-delivered miRNAs via extracellular vesicles is building, although the ultimate impact of this mechanism on the gene expression of many cell types has yet to be determined.

Circulating miRNAs

miRNAs are found free in both plasma and serum and therefore have the potential to serve as biomarkers for diseases. In general, miRNAs are very biochemically stable and compared to mRNAs have superior performance characteristics as biomarkers for disease activity (Kai and Pasquinelli 2010; Scholer et al. 2010; Lu et al. 2008). This stability in the blood is thought to result from the miRNAs being packaged inside extracellular vesicles such as microparticles, liposomes, or complexed with RNA-binding proteins or high density lipoproteins (reviewed in (Creemers et al. 2012)).

Because platelets contain a significant proportion of the cellular fraction of miRNA in blood and because PMPs are the most abundant microparticle, it is thought that a substantial fraction of circulating miRNAs originates from platelets (Teruel-Montoya et al. 2014; Berckmans et al. 2001). Indeed, miRNA profiles of platelets compared to serum or plasma were shown to exhibit a high degree of correlation, suggesting a platelet origin for many of the circulating

miRNAs (Willeit et al. 2013). Along these lines, circulating levels of miRNAs highly expressed in platelets have been found to be associated with myocardial infarction risk, diabetes, and smoking (Badrnya et al. 2014; Duan et al. 2014; Zampetaki et al. 2012).

Other studies have identified an association between circulating miRNAs and the *intermediate trait* of platelet function. Willeit and colleagues found that plasma miRNA levels were lower in healthy subjects taking antiplatelet medication (Willeit et al. 2013; Yu et al. 2013). Zhang et al. found that reduced circulating *miR-223* levels predicted high platelet function in troponin-negative non-ST elevation acute coronary syndrome patients (Zhang et al. 2014). It was also reported that lower plasma *miR-223* is associated with high platelet reactivity to ADP signaling in patients with coronary artery disease, possibly due to *miR-223* regulation of the $P2Y_{12}$ ADP receptor (Chyrchel et al. 2014; Landry et al. 2009). Finally, circulating *miR-126* was found to correlate with circulating P-selectin levels in diabetic patients and this level was sensitive to aspirin treatment, suggesting a platelet origin (de Boer et al. 2013; Shi et al. 2015).

lncRNAs

Long noncoding RNAs (lncRNAs) are defined as ncRNAs longer than 200 nucleotides. A variety of functions have been assigned to lncRNAs including genomic imprinting, differentiation, cancer progression, and others (reviewed in (Satpathy and Chang 2015)). GENCODE lists 15,931 lncRNAs categorized according to genomic location (long intergenic RNAs (lincRNAs), intronic lncRNAs, and antisense RNAs), length, association with protein-coding genes, and DNA elements (St Laurent et al. 2015). lncRNAs can interact with other nucleic acids through base pairing and also with proteins through secondary RNA structures. Therefore, lncRNAs appear to function through RNA–RNA, RNA–DNA, and RNA–protein associations (Guttman and Rinn 2012). Although a relatively small number of lncRNAs have been well characterized, the vast majority have no known function. Londin et al. detected 192 lncRNAs as present in all ten platelet RNA samples analyzed (Londin et al. 2014). There have been a few reports of lncRNAs regulating erythropoiesis, but megakaryopoiesis is unexplored (Paralkar et al. 2014).

Other Platelet ncRNAs

RNA-seq has been performed on short and long RNA platelet transcriptomes from ten healthy young males (Londin et al. 2014). Approximately 3.5 billion sequence reads were obtained, 40 % of which mapped uniquely to the human genome. About 800 miRNAs and 9000 protein-coding mRNAs were detected in the transcriptomes of *each* of the ten subjects. These mRNAs and miRNAs accounted for only slightly more than half of all of the uniquely mapped sequence reads, suggesting the abundant presence of additional ncRNA transcripts. Detailed bioinformatic analyses revealed ~10,000 non-protein-coding transcripts from mRNA-coding loci, ~1500 pseudogene transcripts, ~200 long intergenic noncoding RNAs (lncRNAs) and abundant signal recognition particle RNAs (srpRNAs), small nuclear RNAs (snRNAs), small cytoplasmic RNAs (scRNAs), and pyknons. Lastly, the platelet transcriptome was rich in a variety of distinct repeat element categories, including DNA transposons, long terminal repeat (LTR) retrotransposons, and non-LTR retrotransposons such as long interspersed elements (LINEs) and short interspersed elements (SINEs). This diverse array of ncRNAs is expected to participate in regulating gene expression, although much work is needed to characterize their effects in megakaryocytes and platelets. Circular RNAs, which have the potential to negatively regulate miRNAs by acting as "sponges," have also been identified in platelets (Alhasan et al. 2015; Hansen et al. 2013; Memczak et al. 2013). Alhasan et al. analyzed platelet RNA-Seq data and identified 3162 genes for which circular transcripts were enriched. In addition, they found the platelets contained more circular RNAs as compared to nucleated cells.

miRNAs as Therapeutics or as Therapeutic Targets

There are numerous diseases caused at least in part by dysregulated miRNA or mRNA expression. The in vivo introduction of pre-miRNAs or mature miRNAs are novel therapeutic approaches to either raise abnormally low miRNA levels or reduce elevated target mRNA levels. Alternatively, undesired miRNA activities can be inhibited by antagomirs or locked nucleic acids (LNAs). Antagomirs are short, complimentary antisense molecules that bind to miRNAs; LNAs are chemically modified oligonucleotides that "lock" the nucleic acid in a conformation which stabilizes the duplexes formed with miRNAs (Kurreck 2003). Another approach is the use of miRNA "decoys or sponges." In this case, single-stranded RNAs with multiple binding sites are used to adsorb the miRNA present in the cell, interfering with its normal function (Ebert et al. 2007). Work done in mouse models of disease demonstrates the potential of using miRNA expression-modifying therapeutics to treat a pathologic conditions, such as cancer (Doebele et al. 2010; Anand et al. 2010; Ma et al. 2010) and cardiac disease (Carè et al. 2007; Thum et al. 2008; Bonauer et al.

2009; Bhagat et al. 2013; Garchow et al. 2011; Rayner et al. 2011a, b; Marquart et al. 2013; Wang et al. 2012; Montgomery et al. 2011). Importantly, LNA-based microRNA inhibition was shown to be effective in reducing viral load in a phase 2 human study of chronic hepatitis C virus infection (Janssen et al. 2013). A long-term, follow-up study observed no long-term safety issues (van der Ree et al. 2014).

The ability of miRNA to inhibit gene expression makes it a useful tool to restrict expression in circumstances where it would cause undesired consequences. Even when tissue-preferential promoters are used to direct transgene expression, off-target and deleterious effects have been observed (Follenzi et al. 2004; Mingozzi et al. 2003). The tissue-preferential expression of miRNAs has been exploited to prevent off-target effects in gene therapy studies of mouse models of hemophilia (Brown et al. 2007). Such a gene therapy approach for hematologic diseases requires knowledge of miRNA levels in different hematopoietic lineages. Teruel-Montoya et al. demonstrated that miRNAs that could be used to restrict transgene expression to a particular blood cell type and that reporter gene expression could be regulated by endogenous miRNA levels in a megakaryocytic cell-specific manner line (Teruel-Montoya et al. 2014). These data established the potential for developing gene therapy vectors that exploit hematopoietic lineage-preferential miRNA expression. In addition, engineering target sites for miRNAs absent in megakaryocytes but present in other hematopoietic cell lineages into a therapeutic transgene could permit megakaryocyte-specific transgene expression in platelet-specific diseases of gene hypofunction.

Blood Bank stored platelets translate protein over time (Thon and Devine 2007) and it is conceivable that the active translation of proteins in stored platelets may contribute to reduced platelet function. An intriguing possibility would be to inhibit the translation of apoptotic and antithrombotic regulatory proteins via introduced miRNAs.

Summary and Future Directions

ncRNAs regulate hematopoiesis and megakaryopoiesis, and platelet miRNAs likely (1) alter platelet gene expression, (2) regulate endothelial cell or other cell gene expression via PMP transfer, (3) serve as important disease biomarkers, (4) define the MK lineage in clonal bone marrow diseases, and (5) serve as tools for understanding basic mechanisms of megakaryocyte/platelet gene expression. Genome-wide association studies have identified intergenic loci where the SNP alters miRNA gene sequence and function (Mencia et al. 2009; Modamio-Hoybjor et al. 2004), and it is likely that additional megakaryocyte/platelet disease-producing genetic variants in miRNA biogenesis will be identified. An enormous number of new miRNAs have been identified by using RNA obtained from multiple cell types (Londin et al. 2014). It will be important to develop commercially available tools for studying these novel miRNAs. Notably, new platelet-specific miRNAs were discovered not in the existing reference database. Exciting possibilities for hematopoietic lineage-restricted transgene expression vectors are possible based on the new knowledge of differentially expressed miRNAs among hematopoietic cells. An increasing number of miRNAs targeting multiple mRNAs that regulate a specific signaling or developmental pathway suggest there are "master-miRNAs" that could be manipulated to affect cell physiology. Ongoing in vivo studies that knock-down or replace defective miRNAs are sure to lead to important laboratory tools and novel human disease treatment approaches. For future platelet miRNA biomarker association studies it will be essential to have appropriate samples sizes and analyses that consider confounding variables known to alter miRNA levels, such as age, gender, race, and medications. Lastly, the non-miRNA repertoire of ncRNAs remains a relatively fledgling field and is sure to yield exciting new insights into megakaryocytes and platelet biology in the very near future.

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Implications of Platelet RNA to Vascular Health and Disease

Lauren Clancy and Jane E. Freedman

Abstract

The historical relevance of platelets has focused on their traditional role in the circulation of hemostasis and thrombosis. Recent studies of platelet composition and novel platelet roles in a number of non-hemostatic systemic responses have since broadened our appreciation for the functional capabilities of platelets. One such expansion of our understanding includes the exploration of the platelet's endogenous RNA and its ability to regulate platelet immune and inflammatory processes. RNA profiling of platelet messenger RNA (mRNA) and microRNA (miRNA) revealed distinct expression patterns associated with a number of platelet phenotypes and disease pathologies. A deeper understanding of the genetic material present in platelets endogenously and in disease settings can further elucidate the role platelet RNA plays in thrombosis and the implications of platelet involvement in fields such as cancer, infection, and systemic inflammation.

Introduction

Historical Role of Platelets in Hemostasis

Platelets are vital to the regulation of thrombosis and hemostasis. Upon induction of platelet biogenesis, platelet progenitor cells, megakaryocytes, mature in the bone marrow for 4–5 days (Michelson 2013). Platelet biogenesis occurs when megakaryocytes extend pseudopodia into the blood sinuses of the bone marrow and the force of blood flow sheers fragments off the pseudopodia, resulting in the release of proplatelets and platelets into the bloodstream (Michelson 2013). As platelets move through the bloodstream over the next 7–10 days, they respond to sites of vascular injury (Lackie 2010; Jurk and Kehrel 2005). Receptor recognition, binding, and further platelet activation lead to granule secretion and the initiation of the thrombotic response (Jurk and Kehrel 2005). Vascular and endothelial cells at injury sites need multiple factors to be carefully balanced to bring about normal hemostasis; circulating platelets responding to these sites help maintain this balance. Thrombotic events ensue when the formation of a clot obstructs a vessel.

Alternative Platelet Functions Throughout the Circulation

Platelets' traditional role in hemostasis and their role in the chronic inflammatory disease, atherosclerosis, illustrate the traditional link between platelets and inflammation and have been studied extensively. However, our understanding of platelets and their capabilities has increased alongside our knowledge of platelets' roles in the body's systemic responses. Deeper connections between local inflammation, infection, and cancer progression have all been unearthed in our study of platelet biology. Studies of rheumatoid arthritis sufferers found these patients possess hyperactive platelet microparticles in their joint fluid, linking platelets to local inflammation development (Boilard et al. 2010; Gasparyan et al. 2011). Similarly, platelets have been associated with the systemic response to infection, both viral and bacterial, through their expression of toll-like

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receptors (TLRs), especially toll-like receptor 4 (TLR4) and toll-like receptor 7 (TLR7) (Vieira-de-Abreu et al. 2012; Garraud and Cognasse 2010; Semple and Freedman 2010; Beaulieu and Freedman 2010; Shiraki et al. 2004; Andonegui et al. 2005; Cognasse et al. 2005; Aslam et al. 2006; Freedman et al. 2010; Clark et al. 2007; Ma and Kubes 2008; Koupenova et al. 2014). The expression of both these TLRs on platelets allows platelets to act as sentinels in the blood—early sensors detecting foreign pathogens [lipopolysaccharide expressed on Gram-negative bacteria in the case of TLR4 (Andonegui et al. 2005; Clark et al. 2007) and single-stranded RNA viruses in the case of TLR7 (Koupenova et al. 2014)]. Activation of these sensors allows platelets to induce a number of immune responses, including neutrophil recruitment and bacterial removal, neutrophil extracellular trap (NET) formation, and heterotypic aggregate development (Clark et al. 2007; Ma and Kubes 2008; Koupenova et al. 2014; McDonald et al. 2012). Studies regarding cancer progression have found that select oncology patients suffer from coagulation disorders throughout their disease progression (Sabrkhany et al. 2011). These individuals subsequently face a greater risk both of thrombotic events, such as pulmonary embolism and deep vein thrombosis, and of inconsistencies in normal platelet values, such as platelet counts and volume (Tesselaar et al. 2007; Sun et al. 1979). Platelet factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), have been correlated to the required physiological processes for tumor metastasis, such as angiogenesis and chemotaxis (Mohle et al. 1997; Wartiovaara et al. 1998; Heldin and Westermark 1999; Heldin 2013). These correlations, along with the existence of activated platelets in tumor vasculature, suggest that platelets play a role in developing the essential blood supply for tumor growth (Pinedo et al. 1998; Verheul and Pinedo 2000) and furthering tumor cell metastasis (potentially through concealing tumor development from systemic inflammatory reactions) (Jain et al. 2010). An inverse relationship between platelet inhibition and thrombotic events and tumor growth further supports the role of platelets in tumor development (Ibele et al. 1985; Jurasz et al. 2004; Niers et al. 2007; Wang and Zhang 2008). As our understanding of platelet capability expanded from its function in hemostasis to one in moderating systemic inflammation, immune response, and cancer progression, one uncertainty that emerged was how the anucleate platelet is capable of such versatile functions.

Platelet Content

Initial Platelet Content Analyses

As described, platelet biogenesis occurs through the manual fragmentation of megakaryocytes, and thus, platelets do not contain nuclei and are incapable of transcription. Evaluation of platelet contents however revealed that platelets contain all the components required for signal-dependent translation (Fig. 1) including functional ribosomes, signaling proteins, messenger RNAs (mRNAs), and microRNAs (miRNAs) (Zimmerman and Weyrich 2008; Weyrich et al. 2009; Rowley et al. 2011). The mechanism of platelet biogenesis combined with the lack of transcriptional ability in platelets implied that the platelet profile reflected that of their parental cells and was therefore inherently invariable; however, further analysis of this concept disagreed. Megakaryocytes are capable of transcription and altering their own transcriptome, allowing for the potential to similarly produce diverse platelets under different circumstances. Additionally, it was found that megakaryocytes specifically sorted and packaged material into platelets in the final stages of thrombopoiesis (Cecchetti et al. 2011). A study of matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) revealed that though megakaryocytes expressed ten MMP family members at the mRNA level, only seven of these were found in platelets (Cecchetti et al. 2011). Additionally, one of these transcripts not found in platelets, MMP-2, was expressed at the protein level in platelets (Cecchetti et al. 2011). This suggests that megakaryocytes can selectively sort mRNAs into platelets during platelet biogenesis (Cecchetti et al. 2011), so though platelet contents at biogenesis is derived from megakaryocytes, it may not directly reflect the entire profile of the parental megakaryocytes. These findings suggested an evaluation of the platelet transcriptome, and proteome was necessary to better understand the functional capabilities of platelets, especially under specific physiological settings where an altered platelet profile might be present.

Platelet RNA Content

Initial microarray-based studies estimated only 1500–4000 transcripts in healthy human platelets (Bugert et al. 2003; Gnatenko et al. 2003); however, the development of deep sequencing allowed for full detection of 9500 mRNA transcripts (Rowley et al. 2011, 2012; Bray et al. 2013). Platelets also contain various forms of small RNAs, with miRNAs accounting for 80 % of the small RNA expressed (Ple et al. 2012). MiRNAs, small noncoding RNA, utilize distinct sequence recognition of target mRNAs to control mRNA translation, with each miRNA having the potential to recognize many mRNAs simultaneously and thus providing a great amount of concurrent regulation. The presence of megakaryocyte-derived precursor and mature miRNAs as well as the presence of the machinery necessary to perform pre-miRNA processing in platelets led to complementary research evaluating the platelet miRNA profile (Ple et al. 2012; Denis et al. 2005). This profiling of platelets identified

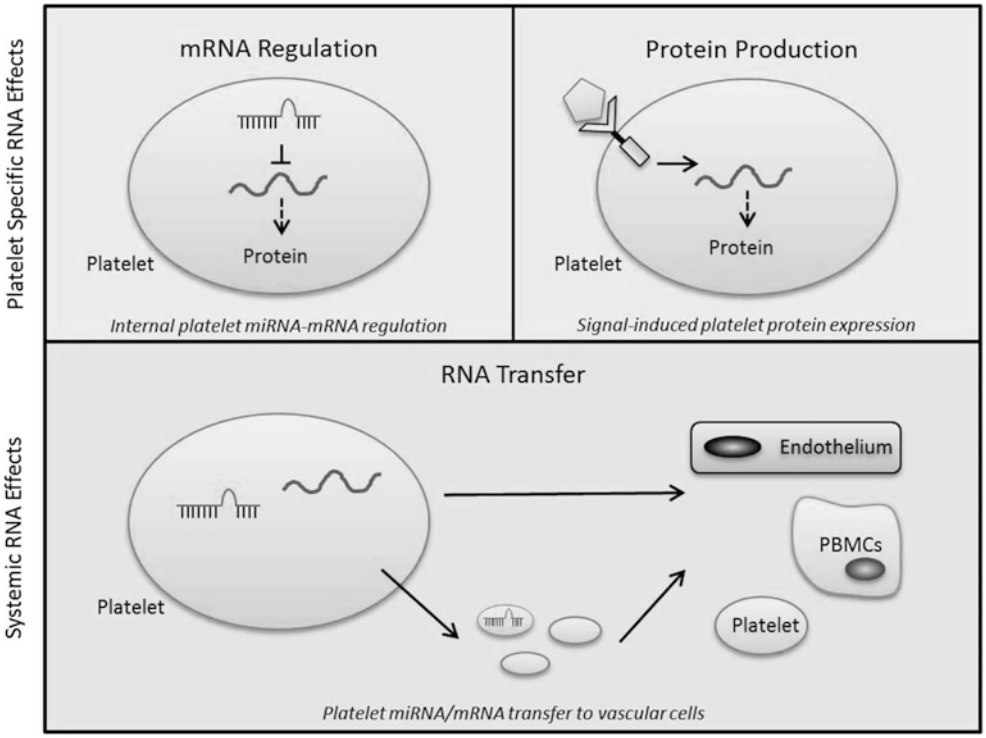

Fig. 1 The role of platelet RNA in the vasculature. Platelets contain broader functions beyond thrombosis including platelet-specific and synthetic RNA functions. Evaluation of platelets reveals that platelets contain all the components required for signal-dependent translation, miRNA–mRNA transcript processes, and functional RNA transfer. Specifically, platelets have the capacity to execute both pre-mRNA processing and protein translation in a signal-dependent manner

532 miRNAs in healthy patient platelets, 40 of which were novel miRNAs, with members of the let-7 family being the most represented (Ple et al. 2012).

Platelet RNA Content and Disease Associations

While initial studies focused on healthy platelet profiling, follow-up focused on correlating platelet RNA profiling to platelet reactivity, species variability, and disease modeling. Differential expression analysis of healthy patient miRNA profiles revealed a correlation between platelet reactivity and miRNA and mRNA expression (Nagalla et al. 2011; Landry et al. 2009). Seventy-four miRNAs were differentially expressed in platelets, with the majority showing increased expression in hyperreactive platelets (Nagalla et al. 2011). This corresponded to an increased differential expression of mRNAs in hyporeactive platelets (Nagalla et al. 2011). Analysis of their findings in hyperreactive platelets to identify differentially expressed increased miRNAs with corresponding decreased target mRNAs revealed 12 miRNA–mRNA pairs, establishing a role for miRNA regulation of mRNA (and thus protein) expression in platelet reactivity (Nagalla et al. 2011). Similarly, an early comparison study between human and mouse platelets evaluated potential roles RNA may play in platelet functional diversity (Rowley et al. 2011). The study identified a large amount of conservation (approximately 4990 transcripts) between species, with 58 % of human platelet transcripts conserved in the mouse platelet profile and 83 % of the mouse profile found in the human profile; however, it also revealed distinct expression variants between the two species which reflected previously described functional differences between human and mouse platelets (Rowley et al. 2011). Specifically, expression of protease-activated receptor (PAR) transcripts in human and mouse platelets directly reflected previously known information about human and mouse platelet signaling (Rowley et al. 2011). This study found the transcript for PAR1, the primary thrombin receptor in human platelets, was expressed in human platelets at 13.5 times the level it is in mouse platelets, and mouse platelets expressed an alternative PAR transcript, PAR3 (Rowley et al. 2011; Darrow et al. 1996; Connolly et al. 1996; Ishihara et al. 1998). This corresponds to and explains previous data showing knockdown of PAR1 in mice having no effect on the mouse platelet thrombin response (Rowley et al. 2011; Darrow et al. 1996; Connolly et al. 1996). Similarly, previous studies showing differential response to platelet-activating factor (PAF) in human and mouse platelets (human platelets responded, mouse platelets did not) are explained by the presence of platelet-activating factor receptor (PTAFR) transcript in only human platelets, not mouse platelets (Rowley et al. 2011; Tsakiris et al. 1999; Terashita et al. 1985). Finally, the presence of CD68 transcript in human platelets and not mouse platelets correlates to CD68 platelet protein expression (Rowley et al. 2011). All together, these studies demonstrate the functional relevance of platelet RNA to platelet reactivity.

The ability of megakaryocytes to alter their transcriptome and preferentially sort platelets led to further evaluation of platelet RNA profiles under various physiological conditions. An abundant amount of correlations exist between platelet RNA expression and physical phenotypes and pathologies. Studies have identified specific differential profiles associated with patient age (129 mRNAs and 15 miRNAs), patient gender (54 mRNAs and 9 miRNAs), and patient race (Simon et al. 2014; Edelstein et al. 2013). Body mass index was significantly ($P < 0.005$) linked to higher expression of 11 of 48 transcripts analyzed in platelets from the Framingham Heart Study, highlighting differential expression of platelet inflammatory transcripts such as intercellular adhesion molecule 1 (ICAM1), S100 calcium-binding protein A9 (S100A9), interleukin 6 (IL6), interleukin 1 receptor 1 (IL1R1), and TLR2 (Freedman et al. 2010).

Similar differential profiles exist under various pathologies as well. Systemic lupus erythematosus (SLE) patients' platelets displayed the type 1 interferon (IFN) gene signature, with increased expression of type 1 IFN-related transcripts (PRKRA, IFITM1, SELP, and/or CD69), especially in patients with a history of vascular disease, myocardial infarction, or arterial or venous thrombosis (Lood et al. 2010). A characterization of sickle cell disease (SCD) is an increased risk of cardiovascular complications specifically linked to increased endothelial damage, inflammation, and platelet activation (Jain et al. 2013). Increased platelet activation directly affects SCD progression through a number of mechanisms, such as creation of microparticles and increased plasma expression of pro-coagulation and adhesion factors (Jain et al. 2013). Coinciding with previous reports linking platelet reactivity to distinct miRNA profiles, a study identified 40 differentially expressed miRNAs (16 upregulated, 24 downregulated) in SCD patient platelets and correlated these patterns to specific miRNA families and resulting regulation of SCD platelet mRNA (Jain et al. 2013).

Unsurprisingly, platelet RNA expression correlations exist for cardiovascular and platelet-related disease as well. RNA comparison of platelets from acute ST-segment elevation myocardial infarction (STEMI) patients and stable coronary artery disease (CAD) patients revealed 54 differentially expressed RNAs, 29 increased and 25 decreased in STEMI patients, with the most significant increased transcripts being CD69 and MRP-14 (Healy et al. 2006). Increased platelet transcript expression of MRP-14 correlated to plasma protein levels of MRP-8/MRP-14, and a prospective case study utilizing the Women's Healthy Study correlated MRP-8/MRP-14 plasma protein levels to incidence of female patients experiencing an initial cardiovascular event (Healy et al. 2006). Thrombocytosis, high platelet counts in the blood, can be differentiated into different classes, specifically essential thrombocythemia (ET), a myeloproliferative disorder, and reactive thrombocytosis (RT) (Gnatenko et al. 2010). Definitive diagnosis of the etiology of thrombocytosis is often difficult, and as the two types' prognoses and associated complications differ, this lack of clear understanding can impede treatment (Gnatenko et al. 2010). RNA sequencing of thrombocytotic patients identified unique RNA profiles of the two phenotypes of thrombocytosis and allowed for selection of an 11-transcript biomarker set which can be used to differentiate RT, ET, and normal patients (Gnatenko et al. 2010). Additional studies by the same group identified a 21-miRNA biomarker set for distinguishing ET patients from normal patients, which could be narrowed to a three-miRNA subset (miR-10a, miR-148a, and miR-490-5p) using statistical analyses (Xu et al. 2012). The identification of these unique mRNA and miRNA signatures to the forms of thrombocytosis may allow for better detection, diagnosis, and treatment in patients. With the presence of unique platelet RNA expression profiles associated with such widespread clinical phenotypes in the realms of disease and inflammation, the study of the functional roles of platelet RNA has developed into an increasingly relevant field of research.

Roles for Platelet RNA

Translational Capacity of Platelet RNA

Initial assumptions centered on platelet RNA being integral to platelet protein production and subsequent platelet alteration in response to their microenvironment (Fig. 1). Platelets have the capacity to execute both pre-mRNA processing and protein translation in a signal-dependent manner (Zimmerman and Weyrich 2008; Denis et al. 2005). It has been demonstrated that lipopolysaccharide signaling in platelets resulted in the processing of pro-IL1β mRNA and the translation and upregulation of IL1β protein, permitting platelet response to bacterial infection (Shashkin et al. 2008). Additionally, platelets possess the ability to process pre-miRNA to mature miRNA, allowing for platelet miRNA regulation of platelet mRNA expression (Denis et al. 2005); this is demonstrated in studies where the existence of a miRNA network affecting expression of the P2Y12 receptor within platelets affected the ability of the platelet to aggregate (Landry et al. 2009). Despite these findings, analysis and comparison of the platelet transcriptome to the platelet proteome revealed that the platelet transcriptome was not completely represented at the proteome level, with estimates ranging from 70 to 90 % coverage (McRedmond et al. 2004; Londin et al. 2014).

Platelet RNA Transfer

Platelets utilize many types of cell-to-cell communication, including direct, moving through the platelet canalicular membrane system, and indirect, discharging microvesicles and exosomes, methods (Risitano et al. 2012; Aatonen et al. 2012; Heijnen et al. 1999; Montecalvo et al. 2012; Valadi et al. 2007; Wahlgren et al. 2012). The possibility of a functional role of platelet RNA being platelet cell communication was hypothesized due to the existence of these cellular communication capabilities and the presence of the unexplained fraction of platelet RNA post-transcriptome-proteome correlation studies. Early research demonstrated platelet RNA transfer through in vitro co-incubation of platelets and vascular cells (Fig. 2), tracking the unique movement of functional platelet-derived transcripts to endothelial and monocyte cells using fluorescence methods and RT-qPCR (Risitano et al. 2012). In vivo infusion experiments utilizing knockout mice supported this phenomenon: transfer of a wild-type TLR2 transcript from wild-type platelets to knockout monocytes was induced through lipopolysaccharide induction of platelet-monocyte interactions (Risitano et al. 2012). These initial findings demonstrated the potential relevance of platelet RNA transfer to elucidating platelet RNA significance.

The role of platelet miRNA in regulating mRNA expression does not account entirely for the functional relevance of platelet miRNA. As mentioned, miRNA have the distinct ability to regulate several target mRNA at once, resulting in potential prolific effects on platelet mRNA levels. This effect can be extended to vascular cells when combined with the phenomenon of platelet RNA transfer. Complimentary to previous analyses of platelet miRNA and their role in STEMI, one study demonstrated that eight miRNAs were significantly downregulated in activated platelets at the thrombus formation of patients with STEMI when compared to the thrombi of non-STEMI patients (Gidlof et al. 2013). Supplementary focus on four of these miRNAs correlated significant increases in their plasma expression to platelet thrombin activation (Gidlof et al. 2013). Additional analysis of STEMI patient peripheral and thrombotic platelets revealed downregulation of these same miRNAs at the site of occlusion as compared to peripheral STEMI or healthy platelets (Gidlof et al. 2013). Further in vitro analysis demonstrated time- and activation-dependent transfer of miRNA from platelets, into platelet-derived microparticles, and then into endothelial cells, utilizing a synthetic exogenous miRNA transfection cell modeling system (Gidlof et al. 2013). This transfer was confirmed in alternative studies analyzing miR-223, the most highly expressed platelet miRNA, and its thrombin-induced release from platelets into platelet-derived microparticles alongside additional confirmation of platelet-derived microparticle uptake by endothelial cells (Laffont et al. 2013). This extension of

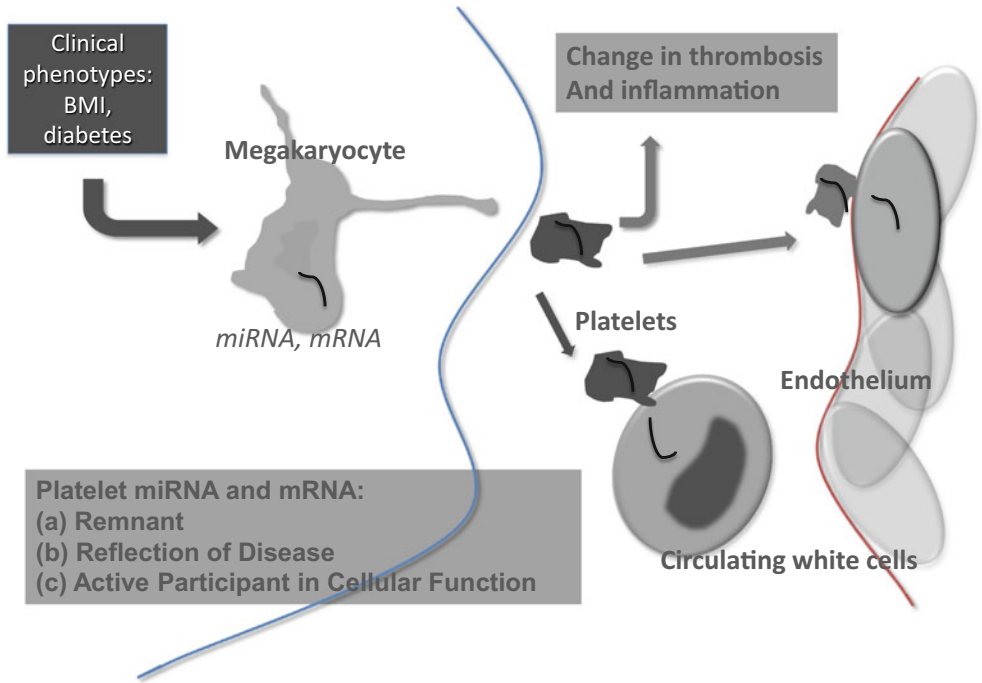

Fig. 2 Platelets and RNA transfer. Platelets play a role in the body's systemic reaction to vascular injury, inflammation, and infection, communicating with other vascular cells by direct transfer of mRNA and miRNA. Research demonstrates that this novel type of cellular communication impacts the recipient cells' phenotypes and may reflect disease states in patients. Different types of RNA may transfer including mRNA and miRNA, and recipients of this transfer process include endothelial cells and white cells

the study of platelet RNA transfer to miRNA transfer further connects platelet RNA to platelet and vascular function.

Integral to the potential relevance of platelet RNA transfer is the capability of platelet RNA to impact its recipient cells through functional regulation, translation, and expression. Three separate labs have confirmed this functional relevance through various modeling systems. The transfer of GFP-labeled vector and its subsequent fluorescent expression from platelet-like particles to monocyte and endothelial cells confirmed the functional relevance of platelet mRNA transfer to vascular cells (Risitano et al. 2012); similarly, the use of targeted luciferase reporter constructs confirmed miRNA transfer and regulation conferred through transfer from platelets to endothelial cells, with one study demonstrating a 30 % downregulation of the targeted mRNA (Gidlof et al. 2013) and another study reporting 44 % downregulation of the target mRNA (Laffont et al. 2013). Similarly, platelet microparticle incubation and subsequent miRNA transfer resulted in >50 % downregulation of miR223-associated endogenously expressed mRNA targets (Laffont et al. 2013). These studies reinforce the notion that platelets—particularly in stimulated environments—can transfer their RNA to other cells of the vasculature and collectively point to a role for platelet transcripts in controlling the expression profiles of other vascular cells. Although the method and in vivo consequences of RNA transfer remain unclear, platelet transcripts seem to play an important role in vascular health beyond traditional clot formation.

Potential Platelet RNA Disease Relevance and Research Applications

As previously outlined, correlations exist between specific platelet profiles and several physiological and disease states. The response of megakaryocytes to their acute environment and pathophysiological alterations in the body may influence the type of platelet produced during thrombopoiesis, with specific platelet transcripts reflecting the overlying condition. New diagnostic and therapeutic tools could arise if the function that these specific transcripts have in the disease state was more fully understood. If these transcripts are specifically sorted by megakaryocytes into platelets in response to a systemic stimulant, it may suggest a role for the transcript in platelet function and disease development which could shed light on the disease itself (such as sorting of a specific signaling transcript into platelets to allow for platelets to respond to a particular form of infection or inflammation occurring systemically). Alternatively, the presence of a specific transcript in platelets under certain conditions or during a disease state may not reflect a role in platelet function and disease development; instead, it may simply represent the upregulation of these transcripts by megakaryocytes responding to stimuli and the subsequent increase in platelet expression due to the mechanism of platelet biogenesis. Though not causally linked to disease development, this scenario still poses advantages to researchers, as platelet transcripts could be used as biomarkers. Conventional biomarker research concentrates on identifying protein biomarkers, but given the availability and accessibility of platelets and the correlation of distinct platelet mRNA and miRNA profiles to physiological conditions, RNA-based biomarkers present in platelets could be utilized to identify disease states, aid research, and develop therapeutic monitoring systems. One such example of therapeutic monitoring has already been described: the use of specific platelet RNA profiling in differentiating the classical phenotypes associated with thrombocytosis. An example of research development through platelet RNA profiling involves gray platelet syndrome (GPS), or platelet alpha-granule deficiency, a bleeding disorder associated with platelets lacking alpha granules (Kahr et al. 2011). GPS platelet RNA sequencing showed abnormal results for a specific transcript, NBEAL2 (Kahr et al. 2011). Further analysis revealed mutations in this gene resulted in abnormal pre-mRNA processing, and this and additional mutations identified in the study were found to be causally linked to disease development (Kahr et al. 2011). Further identification of platelet biomarkers and their correlation to disease states may allow for future development of disease screening, especially relevant for diseases that are difficult to diagnose.

Full elucidation of systemic platelet capabilities fully depends on our total comprehension of the function of platelet RNA. If platelet transcripts directly impact platelet and vascular function through either translational control or RNA transfer, realizing how these transcripts influence disease development will shed light upon both the given disease and possible treatments for it. For example, as described, the correlation between increased presence of MRP-8/MRP-14 protein (and the corresponding increase in MRP-14 transcript) and future cardiovascular events in women (Healy et al. 2006) not only reveals information about the development of cardiovascular disease in women but also potential diagnostic and therapeutic targets. Furthermore, the ability of platelets to transfer RNA to vascular cells also has potential for therapeutic implications. Recent studies have utilized platelet membranes to improve nanoparticle drug delivery (Hu et al. 2015), and once the mechanism and relevance of platelet RNA transfer and its subsequent effect on recipient cells is fully understood, there may be potential to develop platelet RNA transfer into a similar individualized therapeutic delivery system.

Conclusion

The systemic relevance of platelets is no longer limited to their conventional roles in hemostasis and thrombosis. As a result of a number of novel and widespread studies, our understanding of platelet biology has significantly expanded: platelets are now associated with the body's systemic response to vascular injury, inflammation, and infection; our understanding of platelet biology has broadened to include correlations and connections to physiological states, diseases, and cellular communication; and the possibilities for platelet impact on vascular cells' phenotypes and disease and therapeutic development have opened whole new fields of study in platelet functional relevance. Accordingly, a stronger comprehension of platelet RNA transcripts bolsters our understanding of both platelets' conventional thrombotic functions and their nonconventional functions, potentially having extensive implications in a variety of fields.

Take-Home Messages: Implications of Platelet RNA to Vascular Health and Disease

Platelets have a wide variety of RNA including mRNA and miRNA reflecting risk factors and disease.

RNA within platelets is capable of participating in protein production.

MicroRNA in platelets is sensitive to platelet function and may participate in gene expression.

RNA within platelets may be transferred to other vascular cells and participate in regulatory function.

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GTPases

Lucia Stefanini, Robert H. Lee, and Wolfgang Bergmeier

Abstract

GTPases (GTP hydrolases, GTP-binding proteins) are enzymes that direct various cellular functions, including signal transduction, protein biosynthesis, cell division, and transport of vesicles. They are molecular switches that cycle between two conformational states (Fig. 1): the inactive guanosine-5′-diphosphate (GDP)-bound state and the active guanosine-5′-triphosphate (GTP)-bound-state. In the GTP-bound 'on' state GTPases bind effector proteins and generate cellular responses, until GTP hydrolysis returns the switch to the 'off' state. GTP binding and hydrolysis occur in the highly conserved G domain. GTPases are characterized by low intrinsic GDP–GTP exchange and GTP hydrolysis rates; thus, both processes need to be controlled by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) in order for the molecular switch to function at physiologically relevant rates. With few exceptions, GTPases are grouped into two main superfamilies: heterotrimeric G proteins and small GTPases. In this chapter, we will summarize the current state of knowledge about the role of these proteins in the regulation of platelet functional responses, with a particular focus on the tight functional interrelationship between the most well-studied members of the two families.

Heterotrimeric G Proteins

General Aspects

Heterotrimeric GTP-binding proteins, often simply referred to as G proteins, associate with G protein-coupled receptors (GPCRs) in the plasma membrane to relay external signals and convert them into intracellular signaling responses. The G protein heterotrimer is made up of α, β, and γ subunits (Fig. 1A). In humans, there are 16 genes coding for G_α subunits, 5 genes for β subunits, and 14 genes for γ subunits (Milligan and Kostenis 2006). The G_α families ($G_{q/11}$, $G_{12/13}$, G_i, G_s) are grouped based on their structure and functional associations with downstream effectors (Fig. 2). The initial families identified were the cholera toxin-activated, adenylyl cyclase (AC)-stimulating G_s proteins, and the pertussis toxin-sensitive, AC-inhibiting G_i proteins (Milligan and Kostenis 2006). The other major families are the phospholipase C (PLC)-linked G_q proteins (Strathmann and Simon 1990; Taylor et al. 1990) and the RhoGEF-activating

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$G_{12/13}$ proteins (Riobo and Manning 2005). The heterotrimeric complex is comprised of the monomeric G_α subunit in association with the β/γ subunit complex, which forms a structurally stable, functional heterodimer (Fig. 1A). Under basal conditions, G_α is GDP bound and has a high affinity for $G_{\beta\gamma}$ and a low affinity for effector proteins, which stabilizes association of the heterotrimer. This interaction serves multiple purposes. It decreases the intrinsic rate of GDP release from G_α, preventing spontaneous GTP binding and activation (Higashijima et al. 1987). It also assists in localizing G_α to the membrane by association with the highly hydrophobic β/γ complex (Evanko et al. 2001), and it prevents signaling by the β/γ complex, which has been increasingly recognized as a critical component of GPCR signaling (see later) (Khan et al. 2013). Upon ligand binding, the GPCR associates with the G_α subunit and induces a conformational change that triggers the release of GDP (Farrens et al. 1996). As GTP is ~10-fold more abundant in the cytosol than GDP, nucleotide-free G_α now binds GTP and dissociates from the $G_{\beta\gamma}$ subunit. The GTP-bound G_α subunit and the $G_{\beta\gamma}$ unit are then free to interact with downstream effectors (Fig. 1A). Thus, the GPCR serves as a GEF for the α subunit of heterotrimeric G proteins. The inactivation of heterotrimeric G proteins depends on GAP proteins, i.e., proteins that increase the rate of GTP hydrolysis. The prototypic GAPs for G proteins are Regulator of G-protein Signaling (RGS) proteins; platelets express several members of this family, which we will review in more detail later. In the case of $G_{q\alpha}$, additional GAP activity is provided by its downstream target phospholipase C PLC-β (Berstein et al. 1992; Harden et al. 2011). However, the contribution of PLC to $G_{q\alpha}$ inactivation has not been evaluated in platelets and will not be discussed in this chapter.

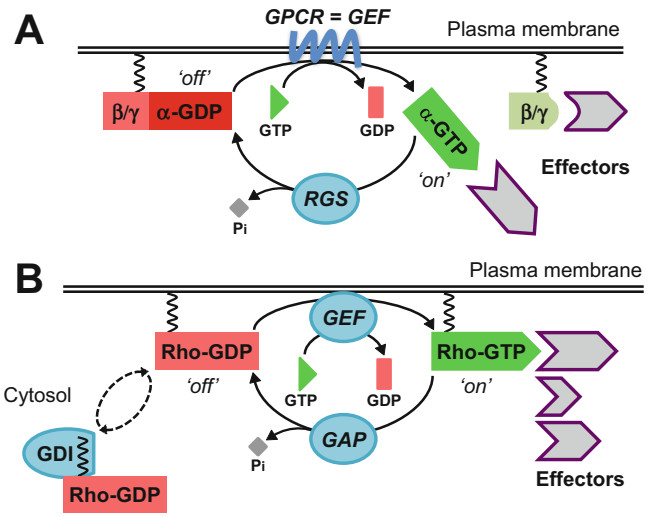

Fig. 1 The GTPase molecular switch. (**A**) Regulation of heterotrimeric G proteins by GPCRs and RGS proteins. Heterotrimeric G proteins are activated by GDP/GTP exchange on their α subunit. This exchange is stimulated by the GPCR, which functions as a guanine nucleotide exchange factor (GEF). GTP loading of the α subunit (*green*) leads to separation of the α and β/γ subunit, which then are free to interact with downstream effectors (*purple*). Inactivation of G_α requires hydrolysis of the γ-phosphate in GTP, a process that is stimulated by RGS (Regulator of G protein Signaling, *blue*) proteins. Inactive, GDP-bound G_α then reassociates with the βγ subunit, completing the inactivation cycle. (**B**) Regulation of the small GTPases by GEFs, GAPs, and GDIs. Small GTPases (RHO is shown as an example) are also activated by GDP/GTP exchange, stimulated by GEFs, and inactivated by GTP hydrolysis stimulated by GAPs (GTPase-activating proteins). In addition to the GDP/GTP switch, the activity of several GTPase families (RHO, RAB, and some RAS) is regulated by GDIs (Guanine nucleotide Dissociation Inhibitor) or GDI-like proteins, which sequester the small GTPases in the cytosol. Active (GTP-loaded) small GTPases are indicated in green, inactive (GDP-loaded) small GTPases are indicated in red, activity regulators are shown in blue, and downstream effectors are in purple. Symbols: GDP (guanosine-5′-diphosphate, green triangle), GTP (guanosine-5′-triphosphate, red rectangle), Pi (inorganic phosphate, gray diamond)

During the course of platelet activation in vivo, primary agonists (thrombin) and released feedback mediators [ADP, thromboxane A_2 (TxA_2)] trigger multiple G-proteins simultaneously, which then act in concert to promote aggregate formation (Broos et al. 2011; Rivera et al. 2009; Stegner and Nieswandt 2011) (Fig. 3). The major GPCRs that mediate G-protein-dependent platelet activation are the thrombin-sensitive protease-activated receptors (PARs), the ADP receptors ($P2Y_1$ and $P2Y_{12}$), and the TxA_2 receptors (TPα and TPβ). GPCRs with less well-characterized contributions to hemostatic plug formation include the serotonin receptor, 5HT-2A, the adrenergic receptor, α2A, and multiple chemokine receptors. Platelet inhibition is achieved through the prostacyclin receptor, IP, also a GPCR. Of the thrombin receptors, human PAR1, PAR4, and mouse PAR4 couple to G_q (Benka et al. 1995; Sambrano et al. 2001; Vaidyula and Rao 2003). These receptors also activate $G_{12/13}$ to induce RhoGEF/RhoA activation and shape change (Klages et al. 1999). The TxA_2 receptor also couples to both G_q and $G_{12/13}$ (Offermanns et al. 1994; Shenker et al. 1991). $P2Y_1$ couples to G_q but not $G_{12/13}$, while $P2Y_{12}$ specifically activates G_i (Jin and Kunapuli 1998). PAR1 can also directly activate G_i (Voss et al. 2007). Initial platelet activation upon exposure to primary agonists (thrombin) is driven by PLC-mediated cleavage of the phospholipid phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and the second messenger diacylglycerol (DAG). Binding of IP_3 to its receptor on the dense tubular system induces the release of the second messenger Ca^{2+} into the cytosol. Ca^{2+} affects integrin-mediated platelet adhesion by activating CalDAG-GEFI, a guanine nucleotide exchange factor for the small GTPase RAP1 (see later). Just like in other cells, Ca^{2+} is also a critical regulator of a variety of enzymes and cytoskeletal proteins that contain calcium-sensitive regulatory domains (Nesbitt et al. 2003). DAG is best known for its role as an activator of protein kinase C (PKC), which in platelets plays a critical role for the release of the second-wave mediators TxA_2 and ADP (Harper and Poole 2010). Engagement of TPα/β and

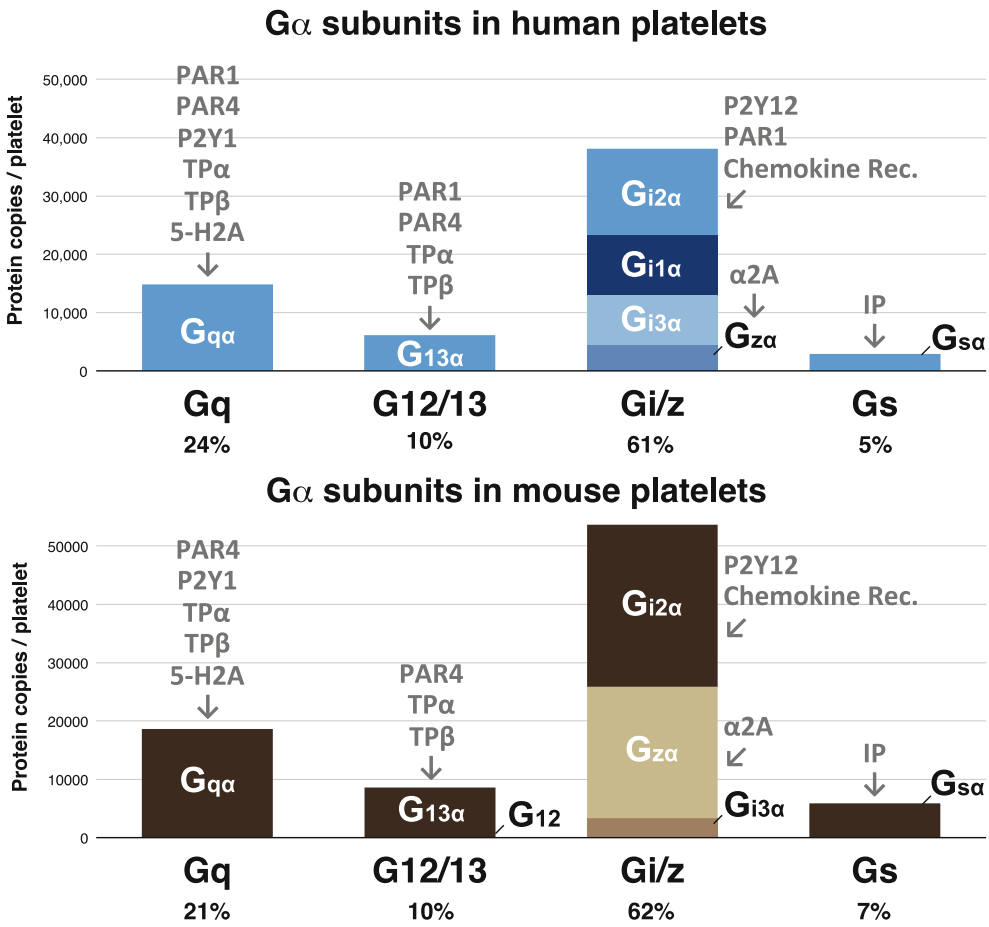

Fig. 2 Expression levels for heterotrimeric G proteins in human and mouse platelets. Graphs show the copy numbers per platelet for the indicated G_α subunits, based on quantitative proteomic analysis; (**top panel**) human (Burkhart et al. 2012) and (**bottom panel**) mouse (Zeiler et al. 2014) platelets. The column height and percentages listed below the columns indicate the relative expression level for each family within the G protein superfamily. Within each column the most abundant isoforms are stacked from top to bottom. G protein-coupled receptors that can activate the respective G proteins are listed in gray

$P2Y_1$ by TxA_2 and ADP, respectively, provides important feedback activation of PLCβ. G_i activation downstream of $P2Y_{12}$ leads to the inhibition of adenylyl cyclase (AC) mediated by $G_{i\alpha}$ and $G_{\beta\gamma}$-mediated activation of phosphoinositide 3-kinase (PI3K). Reduced AC activity leads to lower levels of cyclic AMP and thus reduced negative feedback on platelet activation via protein kinase A (PKA, see later). Signaling downstream of PI3K leads to the inhibition of the RAP1-GAP, RASA3 (Stefanini et al. 2015), and the activation of AKT. Mouse models lacking the major platelet GPCRs have provided valuable information on the role of their associated agonists in hemostasis and thrombus formation. However, the majority of GPCRs couple to more than one family of heterotrimeric G proteins, making it difficult to dissect the phenotype of these mice in regard to the function of individual G proteins (for thorough reviews on platelet GPCRs, see Li et al. 2010; Offermanns 2006). The first section of this chapter will discuss the role of heterotrimeric G protein signaling in platelet activation and thrombus formation, and what we have learned from mouse knockout models and human mutations to put these signaling proteins into the context of bleeding and thrombotic disorders. Key findings from studies in transgenic mouse models and patients with mutations in heterotrimeric G proteins are summarized in Table 1.

Heterotrimeric G Protein Families

$G_{q/11}$ Family

In platelets, G_q is typically the initial heterotrimeric G protein relaying an activation signal upon binding of soluble ligands to GPCRs. Evidence for G_q expression and function was first shown during characterization of the receptors for TxA_2 and thrombin (Hung et al. 1992; Shenker et al. 1991). $G_{11\alpha}$, although expressed in most G_q-expressing tissues, is absent in platelets (Offermanns et al. 1997). G_q signaling is critical for most functional responses in platelets, as it facilitates the activation of PLCβ2/3 and the generation of the second messengers $Ca^{2+}$and DAG. Consistently, platelets from mice lacking $G_{q\alpha}$ exhibit a severe defect in Ca^{2+} mobilization from intracellular stores and in DAG/PKC-mediated granule release in response to thrombin, TxA_2, or ADP. When activated via GPCRs, $G_{q\alpha}$-deficient platelets also show a marked defect in integrin activation and aggregate formation. However, they are able to undergo $G_{12/13}$-mediated shape change upon activation with thrombin or TxA_2 (see later)

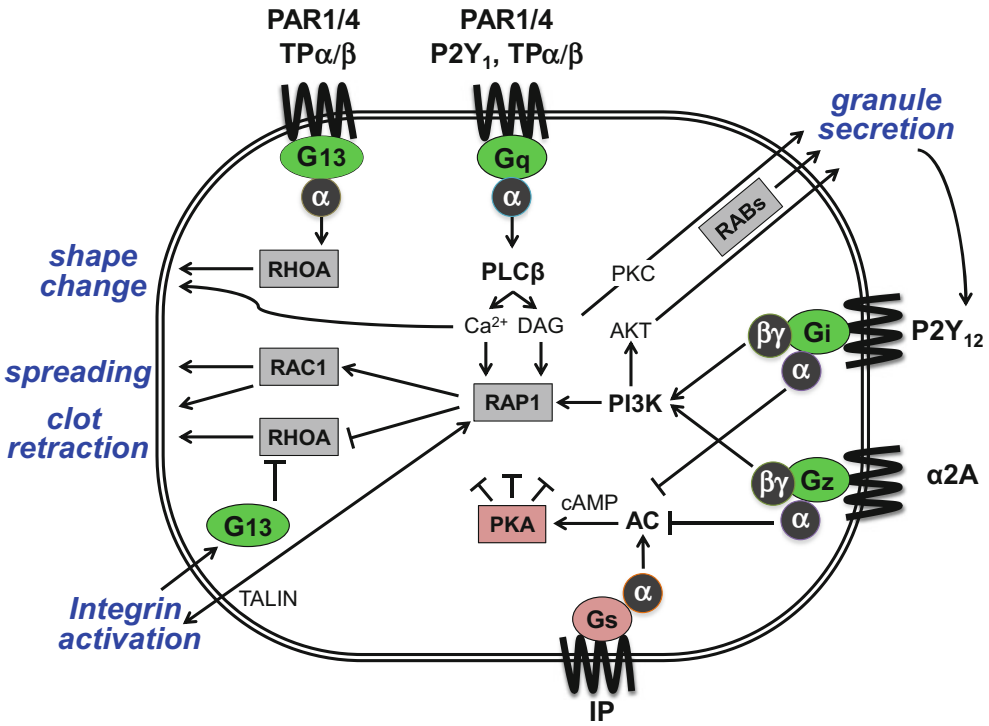

Fig. 3 Heterotrimeric G proteins as key regulators of platelet signaling. This schematic shows the best-characterized interactions between the various G proteins and the main G protein-coupled receptors (GPCRs) on the platelet surface. During the course of platelet activation in vivo, primary agonists (thrombin) and released feedback mediators [ADP, thromboxane (TxA_2)] trigger multiple G-proteins simultaneously, which then act in concert to promote various cellular responses (listed in *blue*). The major GPCRs that mediate G-protein-dependent platelet activation are the thrombin-sensitive protease-activated receptors (PARs), the ADP receptors ($P2Y_1$ and $P2Y_{12}$), the TxA_2 receptors (TPα/β), and the adrenaline receptor (α2A). These receptors activate the indicated heterotrimeric G proteins (*green circles*), leading to dissociation of the subunits and G_α or $G_{\beta/\gamma}$-mediated effects on downstream signaling proteins (figure focused on the interaction with small GTPases, *gray* boxes). Platelet inhibition is achieved through the prostacyclin receptor, IP, and Gs-mediated activation of PKA (*red*). Abbreviations: AC (adenylyl cyclase), Ca^{2+} (calcium ions), DAG (diacyl glycerol), G_q G_{13} G_i (heterotrimeric G proteins), PAR (protease-activated receptor), PI3K (phosphoinositide-3 kinase), PKA (protein kinase A), PKC (protein kinase C)

(Offermanns et al. 1997). A significant defect is also observed when $G_{q\alpha}$-deficient platelets are activated via the collagen receptor, GPVI, a member of the immunoreceptor tyrosine-based activation motif (ITAM) family, likely due to the loss of signal enhancement by the G_q-coupled TP receptor (Packham et al. 1991). Mice deficient in $G_{q\alpha}$ are unable to stop bleeding in a tail clip assay. They are also almost completely protected from collagen/epinephrine-induced thromboembolism (Offermanns et al. 1997) and from thrombosis at the site of severe arterial lesions (Nonne et al. 2005), suggesting a prominent role for G_q signaling in hemostasis and thrombosis. $G_{q\alpha}$-deficient pups also exhibit an increased frequency of intra-abdominal bleeding and perinatal death (Offermanns et al. 1997). Impaired G_q activity is also associated with a bleeding diathesis in humans, and various cellular functions including integrin-mediated aggregation, TxA_2 release, and Ca^{2+} mobilization are markedly impaired in G_q-defective human platelets stimulated via GPCRs (Gabbeta et al. 1997).

G_i Family

Platelets express several G_i family members, including $G_{i2\alpha}$ and $G_{i3\alpha}$. GTP-loaded $G_{i\alpha}$ mediates the inhibition of AC and thus relieves the inhibition of PKA substrates, which include proteins involved in Ca^{2+} mobilization and cytoskeletal rearrangements, as well as small GTPases (Smolenski 2012). $G_{\beta\gamma}$ subunits dissociated from $G_{i\alpha}$-GTP affect platelet activation by associating with multiple effectors including PI3K and PLC. These events are crucial for sustained platelet activation; they are mainly triggered by the binding of the second-wave mediator ADP to its G_i-coupled receptor, $P2Y_{12}$ (Hollopeter et al. 2001; Maayani et al. 2001). The lipid kinase PI3K phosphorylates PIP_2 to phosphatidylinositol 3,4,5-triphosphate (PIP_3), which provides a platform for the binding of proteins that contain a pleckstrin homology (PH) domain to the inner leaflet of the plasma membrane (Lova et al. 2003). These PH domain-containing proteins include AKT, PLC, and BTK, among others (Varnai et al. 2005). In platelets, PI3K-mediated activation of AKT has a well-documented role in granule secretion and cellular adhesion (Kim et al. 2004;

Table 1 Platelet and hemostasis phenotypes in mice and humans lacking components of heterotrimeric G protein signaling

Protein/gene	Platelet phenotype	Hemostasis/thrombosis phenotype	Refs
Mouse KO model			
Gq	↓↓↓ IP_3 production, Ca^{2+} flux, secretion and aggregation in response to thrombin, TxA_2, ADP; ↔ shape change and RhoA activation in response to thrombin and TxA_2	↑↑↑ Bleeding time; ↓↓↓ thromboembolism induced by collagen/epinephrine, ↓↓↓ arterial thrombosis	Offermanns et al. (1997), Nonne et al. (2005)
Gi1	Not detected in mouse platelets		Jantzen et al. (2001)
Gi2	↓↓ Aggregation and inhibition of cAMP production in response to ADP, thrombin; ↓↓ fibrinogen binding in response to ADP and low dose thrombin; ↔ shape change	↑↑↑ Bleeding time; ↓↓ formation and stability of arterial thrombi	Jantzen et al. (2001), Devanathan et al. (2015), Yang et al. (2002)
Gi3	No defect	↔ Bleeding time	Devanathan et al. (2015), Yang et al. (2002)
Gz	↓↓ Aggregation and inhibition of cAMP production in response to epinephrine; ↑ basal cAMP levels; ↔ aggregation and secretion to other agonists	↑↑ Bleeding time; ↓↓ thromboembolism; ↔ thromboembolism	Yang et al. (2000, 2002), Kelleher et al. (2001)
G12	No defect	↔ Bleeding time	Moers et al. (2003)
G13	↓↓ Aggregation and secretion in response to thrombin and TxA_2; ↓↓ shape change and RhoA activation	↑↑ Bleeding time; ↓↓↓ arterial thrombosis induced by carotid artery ligation	Moers et al. (2003)
RGS2	↔ PGE1-induced inhibition of collagen-induced aggregation; ↔ thrombus formation in whole blood perfusion assay	↔ Thrombosis induced by $FeCl_3$ exposure	Banno et al. (2012)
RGS18	↑↑ Aggregation in response to thrombin and TxA_2; ↑ integrin activation in response to TRAP	↓↓ Bleeding time; ↑↑↑ thrombosis induced by $FeCl_3$ formation; ↑↑ thrombosis induced by arterio-venous shunt	Delesque-Touchard et al. (2014), Alshbool et al. (2015)
Human mutations			
Gq [patient with <50 % normal Gq protein levels]	↓ Aggregation and secretion in response to ADP, epinephrine, collagen, TxA_2, PAF; ↓↓ TxA_2 production in response to thrombin and ADP; ↓↓ Ca^{2+} flux in response to thrombin and ADP; ↓↓ integrin activation in response to TRAP, ADP, PAF	↑↑ Bleeding time	Gabbeta et al. (1997)
Gi1 [patient with 25 % Gi1 protein expression]	↓ Aggregation in response to ADP and epinephrine; impaired suppression of forskolin-induced cAMP production by thrombin, ADP, epinephrine; ↔ aggregation and shape change in response to collagen, thrombin, PMA	↑ Bleeding tendencies	Patel et al. (2003)
Gs [patient with compound heterozygous mutations in the GNAS1 cluster]	↓↓↓ PGE1 and prostacyclin-induced inhibition of collagen-induced aggregation	↔ Bleeding time; ↑ PFA closure time for collagen/epinephrine and collagen/ADP	Freson et al. (2008)
RGS2 [three related patients with heterozygous G23D mutation]	↓↓ cAMP production in response to prostacyclin, ↓↓ PGE1-mediated inhibition of collagen-induced aggregation	No defects	Noe et al. (2010)

Symbols: increase—↑ mild, ↑↑ moderate, ↑↑↑ severe; decrease—↓ mild, ↓↓ moderate, ↓↓↓ severe; ↔ no change
IP_3 inositol triphosphate, *Ca^{2+}* calcium, *TxA_2* thromboxane A_2, *ADP* adenosine diphosphate, *cAMP* cyclic adenosine monophosphate, *PGE1* prostaglandin E1, *$FeCl_3$* ferric chloride, *PAF* platelet activating factor, *TRAP* thrombin receptor activating peptide, *PMA* phorbol myristate acetate, *PFA* platelet functional assay

Woulfe 2010). PI3K signaling also plays a critical role for sustained RAP1 activation, a process required for prolonged integrin activation and the formation of stable platelet aggregates (Cifuni et al. 2008; Stefanini et al. 2015). Specifically, PI3K activity mediates the inactivation of RASA3, a RAP1-GAP that is required to keep circulating platelets in a resting state (see later). $G_{i2\alpha}$ seems to be the predominant $G_{i\alpha}$ protein in platelets. While loss of $G_{i3\alpha}$ in mice has no discernable effects on platelet function, $G_{i2\alpha}$ deficiency leads to impaired ADP-dependent inhibition of AC and reduced platelet activation in response to ADP and low concentrations of thrombin (Jantzen et al. 2001). In vivo, loss of $G_{i2\alpha}$ impairs

the formation and stability of arterial thrombi (Devanathan et al. 2015), a phenotype that is similar to that of mice deficient in $P2Y_{12}$ (Andre et al. 2003). While $G_{i1\alpha}$ has not been detected in murine platelets (Jantzen et al. 2001), it is expressed in human platelets, and decreased expression of $G_{i1\alpha}$ was detected in a patient with reduced ADP- and epinephrine-induced platelet aggregation and a tendency for bleeding (Patel et al. 2003). Patients with mutations in the more highly expressed $G_{i2\alpha}$ and $G_{i3\alpha}$ have not been described. However, the hemostatic effects of $G_{i\alpha}$ inhibition can be observed with pharmacological inhibition of $P2Y_{12}$ by thienopyridines, a widely used class of antiplatelet drugs (Foster et al. 2001; Hollopeter et al. 2001). These drugs act to destabilize thrombus formation by preventing $P2Y_{12}$ activation and G_i signaling. Both $P2Y_{12}$ inhibitors and mutations in $P2Y_{12}$ lead to an increased risk of bleeding in humans and mice (Getz et al. 2014; Hollopeter et al. 2001).

$G_{z\alpha}$, another G_i family member, associates with the adrenergic receptor for epinephrine (α2A) in platelets (Shenker et al. 1991). While epinephrine alone does not cause platelet aggregation, it is able to enhance integrin activation in the presence of other stimuli (Yang et al. 2002). Interestingly, loss of $G_{z\alpha}$ leads to impaired platelet function (Yang et al. 2000) and mice deficient in $G_{z\alpha}$ exhibit a prolonged bleeding time and a reduction in thrombus stabilization, suggesting that the enhancing effect of epinephrine plays a role in vivo during hemostasis (Kelleher et al. 2001; Pozgajova et al. 2006; Yang et al. 2000).

Finally, limited evidence suggests that $G_{i\alpha}$ functionally couples to PAR1 (Voss et al. 2007) and to chemokine receptors in platelets (Abi-Younes et al. 2000). Platelets express a variety of chemokine receptors (Clemetson et al. 2000), but our knowledge of the role of these receptors in platelet function is limited compared to receptors involved in aggregation. Chemokine binding to platelets has a weak activating effect, but can potentiate activation induced by other agonists, similar to what is seen after engagement of other $G_{i\alpha}$-coupled receptors (Schafer et al. 2004; Walsh et al. 2015). G_i signaling by chemokines likely plays a more critical role in chronic vascular disease such as atherosclerosis (Chatterjee et al. 2015; Gleissner et al. 2008), where, for example, reduced platelet expression of the chemokine receptors for SDF-1α, CXCR4, and CXCR7, is associated with lower mortality in patients with coronary artery disease (Rath et al. 2015).

$G_{12/13}$ Family

During adhesion, platelets undergo shape change from their discoid shape in circulation to a flat, expanded cell, increasing the available surface area for binding at the site of vessel injury. Shape change in platelets depends on the rearrangement of the cytoskeleton, formation of new actin filaments for filopodia extension, and outward expansion of the open canalicular system (Bearer et al. 2002). Granules also become centrally localized to facilitate efficient secretion (Fitch-Tewfik and Flaumenhaft 2013). Soluble agonists that can induce shape change include thrombin, TxA_2, and ADP. Stimulation of PAR1, PAR4, or TP induces shape change via direct engagement of $G_{12/13\alpha}$ proteins. Insoluble agonists, such as collagen, indirectly induce shape change by promoting TxA_2 production and release. Platelets express both family members, $G_{12\alpha}$ and $G_{13\alpha}$ (Offermanns et al. 1994). However, $G_{13\alpha}$ seems to be the primary family member functioning in platelets, as no defects in platelet function were observed in $G_{12\alpha}$-deficient mice (Moers et al. 2003). $G_{13\alpha}$-mediated induction of shape change occurs at agonist concentrations below the threshold for aggregation and does not require an increase in the intracellular Ca^{2+} concentration (Rink et al. 1982). $G_{13\alpha}$ couples to RHO-GEFs, such as p115RhoGEF, which activate RHOA (Kozasa et al. 1998). RHOA activates Rho-associated protein kinase (ROCK), which inhibits myosin light chain (MLC) phosphatase and enhances MLC phosphorylation and MLC-dependent contraction to stimulate shape change and granule secretion (Klages et al. 1999). In addition to shape change, $G_{13\alpha}$ contributes to integrin outside-in (Gong et al. 2010) and inside-out (Srinivasan et al. 2015) signaling, as well as TxA_2 generation (Kim et al. 2013a). Consistent with this variety of platelet defects observed in vitro, mice deficient in $G_{13\alpha}$ exhibit a marked impairment in hemostatic plug formation and carotid artery thrombosis (Moers et al. 2003).

G_s Family

As outlined earlier, platelets contain a very sensitive and powerful signaling machinery that facilitates their adhesion to sites of injury under conditions of high shear stress. To avoid unwanted activation, circulating platelets are exposed to inhibitors that are primarily released by the healthy endothelium. The two major inhibitory factors are nitric oxide and prostacyclin (prostaglandin I_2, PGI_2) (Jin et al. 2005). Nitric oxide is able to diffuse across the plasma membrane to directly activate its target, the soluble NO-sensitive guanylyl cyclase (sGC). Activated sGC leads to the formation of cyclic GMP (cGMP), a potent activator of protein kinase G (PKG) (Smolenski 2012). PGI_2 binds to its receptor (IP) on the platelet surface and activates $G_{s\alpha}$, leading to increased activity of AC and cAMP production (Smolenski 2012). While in most cell types cAMP is stimulatory, in platelets cAMP leads to PKA-mediated phosphorylation of multiple proteins and inhibition of platelet activation and adhesion, irrespective of the agonist used to stimulate the cells (Beck et al. 2014). Examples for PKA substrates include small GTPases such as RAP1B, IP_3 receptors, and various actin-binding proteins (Smolenski 2012).

To turn off inhibitory signaling, cAMP is degraded by phosphodiesterases and $G_{s\alpha}$ signaling is turned off by RGS2 (see later) (Smolenski 2012). Loss of PGI_2-dependent $G_{s\alpha}$

signaling in mice lacking the IP receptor leads to enhanced platelet activation, and $G_{s\alpha}$ signaling seems to be particularly important to counteract TxA_2-induced activation (Cheng et al. 2002). In humans, G_s hypofunction leads to platelet hyperreactivity to various agonists, insensitivity to $G_{s\alpha}$ stimulation, and a thrombotic phenotype (Freson et al. 2008). In contrast, a gain-of-function mutation in $G_{s\alpha}$ causes elevated cAMP levels and an increased susceptibility to bleeding (Van Geet et al. 2009). Drugs that increase cAMP levels have inhibitory actions on platelet function and are used clinically in the treatment of stroke (Gresele et al. 2011). However, the reverse is not true; decreasing cAMP levels alone does not induce platelet activation.

Negative Regulation by RGS Proteins

As outlined earlier, stimulation of G_s signaling by mediators released from the healthy vasculature is critical to keep circulating platelets in a quiescent state. At sites of vascular injury, additional negative feedback is required to dampen platelet activation and to prevent uncontrolled thrombus growth. RGS proteins play an important role in this process, as they function as GAPs to promote GTP hydrolysis and terminate G_α signaling. In humans, there are at least 37 genes for RGS proteins, many of which were detected at the mRNA or protein level in platelets (Bodor et al. 2004; Kim et al. 2006). One of the first studies to examine the role of RGS proteins in platelet function was performed using mice expressing a mutant $G_{i2\alpha}$ protein which is unable to interact with RGS proteins. Platelets from these mice show enhanced aggregation and increased platelet adhesion at the site of vascular injury in vivo (Signarvic et al. 2010). Proteomics and biochemical studies have shown that RGS10 and RGS18 are the most highly expressed RGS proteins in human platelets, and that they are involved in regulating platelet activation by thrombin receptors (Burkhart et al. 2012; Garcia et al. 2004; Zeiler et al. 2014). While RGS10 is widely expressed, RGS18 is enriched in platelets and megakaryocytes, as well as other cells of the hematopoietic lineage. Loss of RGS18 in mice leads to platelet hypersensitivity to activation, increased thrombus formation, and mild thrombocytopenia (Alshbool et al. 2015; Delesque-Touchard et al. 2014). The phenotype of mice deficient in RGS10 has yet to be determined. In resting platelets, RGS10 and RGS18 are sequestered in a complex that includes the scaffold protein spinophilin. Upon platelet activation with thrombin or TxA_2, spinophilin is dephosphorylated causing the release of the RGS protein from the complex (Ma et al. 2012). RGS18 also complexes with the scaffold protein 14-3-3. In this complex RGS18 has a decreased affinity for G_α, and the RGS18/14-3-3 complex is upregulated upon activation by thrombin and TxA_2 to promote extended $G_{q\alpha}$ signaling (Gegenbauer et al. 2012). The balance of free and complex-associated RGS18 is tightly controlled to regulate platelet activation both in the absence and presence of receptor agonists, and implicates RGS18 as a potential therapeutic target to control the status of platelet activation. RGS2 has also been reported to play a role in regulating platelet activation by GPCRs. While a mutation in RGS2 in humans causes impaired platelet G_s signaling due to constitutive association between $G_{s\alpha}$ and RGS2, loss of RGS2 in mice had no apparent effect on thrombus formation ex vivo or in vivo, suggesting potential compensation by other family members in mice (Banno et al. 2012; Noe et al. 2010).

Small GTPases

General Aspects

Small GTPases (also known as Small G proteins or Ras-related GTPases after their founding member) form an independent superfamily within the larger class of regulatory GTPases. They are monomers, similar in biochemistry to the heterotrimeric G protein α subunits (Fig. 1), and consist of a highly conserved globular structure of 20–25 kDa that includes two highly flexible motifs (switch regions 1 and 2). These motifs change conformation when GTP is bound and engage the effectors in the cell. The vast majority of small GTPases are posttranslationally modified by lipid anchors (farnesyl, geranylgeranyl, palmitoyl, myristoyl, or acetyl groups) that enable the association to cell membranes.

Small GTPases possess low intrinsic GTP hydrolysis and GDP/GTP exchange activities, thus GDP/GTP cycling needs to be controlled by GEFs and GAPs (Bos et al. 2007; Cherfils and Zeghouf 2013). Some members of this superfamily, such as RHO, RAB, and few RAS GTPases underlie additional regulatory activity by *guanine nucleotide dissociation inhibitors* (GDIs) or GDI-like molecules, proteins that mask the lipid group and sequester the inactive GTPases in the cytosol (Fig. 1B).

In humans, the small GTPase superfamily includes 153 members and is divided into five branches based on sequence and functional similarities (Wennerberg et al. 2005): RAS (cell proliferation, growth, adhesion), RHO (cytoskeletal dynamics), RAB (membrane trafficking), ARF (vesicular transport and actin remodeling), and RAN (nuclear transport and microtubule regulation). Despite the highly conserved structure, variations in sequence, posttranslational modifications, subcellular localization, and upstream regulatory proteins allow this large family to control a wide range of cellular responses. By expressing a defined set of small GTPases (Fig. 4) and upstream regulatory proteins, platelets acquired a unique signaling machinery that is crucial for their role in hemostasis and wound healing. Our understanding of

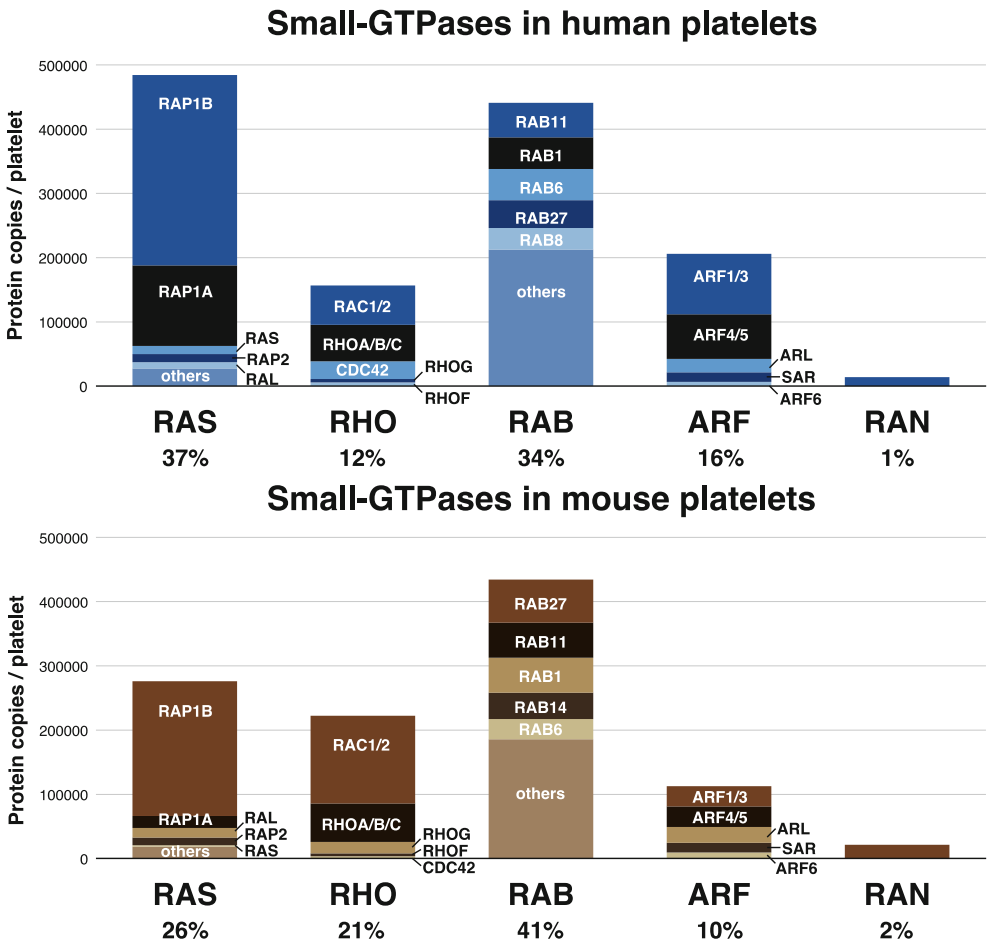

Fig. 4 Expression levels for small GTPases in human and mouse platelets. Graphs show the copy numbers per platelet for the indicated members of the RAS superfamily, based on quantitative proteomic analysis; (**top panel**) human (Burkhart et al. 2012) and (**bottom panel**) mouse (Zeiler et al. 2014) platelets. Each column corresponds to a specific family of small GTPases (RAS, RHO, RAB, ARF, RAN). The column height and percentages listed below indicate the relative expression level for each family within the RAS superfamily. Within each column the most abundant isoforms are stacked from top to bottom

this complex small GTPase signaling network, however, is still incomplete. This part of the chapter summarizes the current state of knowledge about the role of this vast superfamily of proteins in the regulation of platelet functional responses (Fig. 5), with particular focus on the most studied members of the family. Key findings from studies in transgenic mouse models with defects in small GTPase signaling are summarized in Table 2.

Small GTPase Families and Subfamilies

RAS Family

The RAS (*Rat sarcoma*) GTPase family (36 members in mammals) comprises major regulators of cell growth, differentiation, adhesion, and survival and consists of six subfamilies: RAS (cell proliferation, differentiation, and growth), RAP (cell adhesion, exocytosis, cytoskeletal dynamics), RAL (exocytosis), RAD (function unknown), RIT (function unknown), and RHEB (mTOR signaling).

RAP Subfamily

The most highly expressed small GTPases in platelets are members of the RAP subfamily, which are best known for their role in regulating adhesion molecules, the actin cytoskeleton, and MAP kinase cascades. The RAP subfamily consists of five members: two RAP1 proteins (1A and 1B) and three RAP2 proteins (2A, 2B, and 2C), with RAP1A and RAP1B being among the most abundant signaling proteins in platelets (Fig. 4) (Burkhart et al. 2012; Rowley et al. 2011; Simon et al. 2014).

Studies in other cells suggest that RAP1 and RAP2, which share 60 % sequence homology, control distinct cellular responses by signaling through different pools of downstream effectors (Machida et al. 2004; Nonaka et al. 2008; Pannekoek et al. 2013; Taira et al. 2004). Protein and RNA profiling (Burkhart et al. 2012; Rowley et al. 2011; Simon et al. 2014) indicate that platelets express a significant amount of RAP2B (Ohmstede et al. 1990), RAP2C (Paganini et al. 2006), and RAP2A, but RAP2B is the only isoform that has been detected by western blotting (Torti et al. 1993). Compared to RAP1, RAP2 displays a distinct subcellular distribution due to different posttranslational modifications (Canobbio et al. 2008) and in nonstimulated platelets shows elevated GTP loading (Greco et al. 2004; Stefanini et al. 2012), possibly because of a lower

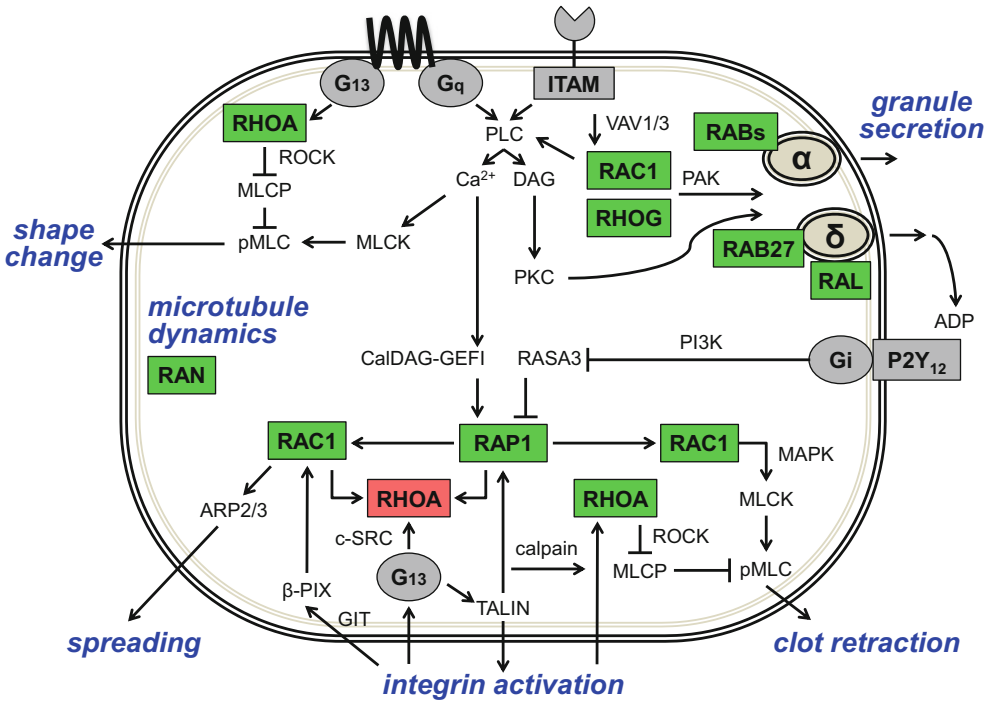

Fig. 5 Small GTPase signaling network in platelets. Active (GTP-loaded) small GTPases are indicated in green, while inactive (GDP-loaded) small GTPases are shown in red. Agonist receptors and heterotrimeric G proteins are shown in gray. Important signaling molecules upstream and downstream of the respective small GTPases are also shown. Abbreviations: α (α-granules), ADP (adenosine diphosphate), ARP2/3 (actin nucleating complex), β-PIX (RAC-GEF), CalDAG-GEFI (RAP-GEF), Ca^{2+} (calcium ions), calpain (calcium-dependent protease), DAG (diacyl glycerol), δ (dense granules), ITAM (immunoreceptor tyrosine-based activation motif-coupled receptors), GPCR (G protein-coupled receptors), G_q G_{13} G_i (heterotrimeric G proteins), GIT (ARF-GAP), ROCK (RHO-associated protein kinases), MAPK (mitogen-activated protein kinases), MLCP (myosin light chain phosphatase), MLCK (myosin light chain kinase), $P2Y_{12}$ (G_i-coupled receptor for ADP), PAK (p21-activated kinase), PI3K (phosphatidylinositide 3-kinases), PKC (protein kinase C), PLC (phospholipase C), pMLC (phosphorylated myosin light chain), RASA3 (RAS/RAP-GAP), c-SRC (tyrosine-protein kinase), TALIN (cytoskeletal protein that mediates RAP1-dependent integrin activation), VAV1/3 (RAC-GEFs)

sensitivity toward GAP-mediated inactivation (Ohba et al. 2000). However, little is known about RAP2 function in platelets and more detailed in vivo studies are lacking.

The high expression of RAP1-GTPases in platelets was recognized in the early 1990s (Kawata et al. 1989; Siess et al. 1990). Subsequent studies established a strong correlation between RAP1 activation and integrin-mediated adhesion upon cellular stimulation (Bos et al. 2003; Caron 2003; Caron et al. 2000; Katagiri et al. 2000; Reedquist et al. 2000). The first genetic evidence confirming a crucial role for RAP1 and its upstream regulator, CalDAG-GEFI, in platelet biology was provided by Shattil and colleagues in engineered murine megakaryocytes (Bertoni et al. 2002; Eto et al. 2002). Shortly thereafter mice deficient in RAP1B (Chrzanowska-Wodnicka et al. 2005) or CalDAG-GEFI (Crittenden et al. 2004) provided definitive proof that integrin activation in platelets is strongly dependent on this signaling pathway. Consistently, both *Rap1b*$^{-/-}$ and *Caldaggef1*$^{-/-}$ mice exhibit a significant defect in hemostasis and a strong protection from experimental thrombosis. These studies also demonstrated that platelet function is more severely impaired in *Caldaggef1*$^{-/-}$ mice compared to *Rap1b*$^{-/-}$ mice, and that the activation of RAP1 and $\alpha_{IIb}\beta_3$ is only partially impaired in *Caldaggef1*$^{-/-}$ platelets. Together, these studies suggested that (1) RAP isoforms other than RAP1B and/or RAP-independent processes contribute to integrin signaling in platelets, and (2) CalDAG-GEFI is not the only regulator of RAP1 activity in platelets.

CalDAG-GEFI is the most abundant RAP-GEF identified in platelets and the only one that has been detected so far by western blotting. Its catalytic activity is regulated by structural rearrangements induced by two EF-hand-binding domains with high affinity ($K_D = 80$ nM) toward Ca^{2+} (Iwig et al. 2013). Consistent with this very high sensitivity toward minor changes in the cytoplasmic Ca^{2+} concentration, platelets from *Caldaggef1*$^{-/-}$ mice (Crittenden et al. 2004) or from patients expressing an inactive CalDAG-GEFI mutant (Canault et al. 2014) fail to aggregate in response to calcium ionophore stimulation and do not respond to threshold doses of physiological agonists. Furthermore, platelets with impaired CalDAG-GEFI function exhibit a delayed activation response to high doses of strong agonists. In vivo,

Table 2 Thrombosis and hemostasis phenotype of mice with defects in small GTPases signaling in platelets

Mouse model	Platelet count/ size	Platelets main features	Secretion	Aggregation	Cytoskeletal dynamics	Thrombosis	Hemostasis	Ref
Rap1b−/−	Normal	Partial integrin activation defect to all agonists	↓ Dense, ↓ alpha	↓ Reduced at LD, normal at HD	↓ Clot retraction, ↓ lamellipodia	↓↓ Nonocclusive 3D thrombi	↑ Bleeding time	Chrzanowska-Wodnicka et al. (2005), Zhang et al. (2011)
Caldaggef1 −/−	Normal	Marked integrin activation defect to most agonists, insensitive to Ca^{2+}, slow activation	↓ Dense, ↓ alpha	↓↓ Abolished at LD, delayed at HD	↓ Clot retraction, ↓ lamellipodia	↓↓↓ 3D thrombi only at low shear rate	↑↑↑ Bleeding time	Crittenden et al. (2004), Stolla et al. (2011a, b), Stefanini et al. (2012)
Rasa3^{hlb}	↓↓↓ Count, ↑↑↑ size	Hyperactivation, short half-life	Normal	↑↑ Independent of $P2Y_{12}$ feedback		Not determined due to severe thrombocytopenia	More stable hemostatic plug	Stefanini et al. (2015)
RhoA$^{fl/fl}$ Pf4Cre^{+}	↓ Count, ↑↑ size	Impaired cytoskeleton-mediated contraction	↓ Alpha	↓ Particularly downstream of G_{13}-coupled receptors	↓ Clot retraction, ↓ shape change	↓↓ Unstable thrombi	↑ Bleeding time	Pleines et al. (2012), Suzuki et al. (2013)
Rac1$^{fl/fl}$ Mx1-Cre^{+} and Rac1$^{fl/fl}$ Pf4-Cre^{+}	Normal	Impaired cytoskeleton-mediated extension, ↓ $PLC\gamma_2$ activation	↓ Dense, ↓ alpha	↓ Particularly downstream of ITAM-coupled receptors	↓ Clot retraction, ↓ lamellipodia	↓↓ Unstable thrombi	↑ Bleeding time	McCarty et al. (2005), Akbar et al. (2007), Pleines et al. (2009), Delaney et al. (2012)
Cdc42$^{fl/fl}$ Mx1-Cre^{+}	↓ Count	Filopodia impaired on FBN and CRP	↓ Dense, ↓ alpha	↓ CRP, COLL, THR	↓ Filopodia, ↓ lamellipodia		↑ Bleeding time	Akbar et al. (2011)
Cdc42$^{fl/fl}$ Pf4-Cre^{+}	↓ Count, ↑ size	Filopodia impaired only on VWF	↑ Dense, ↑ alpha	↑ Enhanced at LD COLL	↓ Filopodia on VWF only	↑ Accelerated thrombus formation	↑ Bleeding time	Pleines et al. (2010)
Rac1$^{fl/fl}$ Cdc42$^{fl/fl}$ Pf4-Cre^{+}	↓↓ Count, ↑↑ size	Aberrant platelet production due to defective microtubule dynamics			↓ Lamellipodia, no microtubules in marginal band		↑↑↑ Bleeding time	Pleines et al. (2013)
RhoG−/−	Normal	Secretion defect in response to CRP	↓ Dense, ↓ alpha	↓ to CRP, rescued by ADP	Normal	↓ Smaller thrombi	Normal	Kim et al. (2013a, b), Goggs et al. (2013a, b)
RhoF−/−	Normal	None	Normal	Normal	Normal	Normal	Normal	Goggs et al. (2013a, b)
Larg−/−	Normal	Agonist-induced RHOA activation not affected	↓ Dense	↓ U46 and PAR4p	Normal	↓ Smaller thrombi only at LD $FeCl_3$		Williams et al. (2015)
Ophn1−/−	Normal	↑ RHOA, CDC42, RAC1 activation	↑ Dense, ↑ alpha	Normal aggregation, ↑ FBN binding	↑ Lamellipodia, ↑ filopodia	↑ Shorter occlusion time	↓ Bleeding time	Fotinos et al. (2015)
Vav1−/− −Vav3−/−		↓ $PLC\gamma_2$ activation		↓ COLL, CRP, RHOD	↓ Spreading on COLL and FBN			Pearce et al. (2004, 2007), Suzuki-Inoue et al. (2006)
P-Rex1−/−	Normal		↓ Dense	↓ Reduced at LD, U46 and THR			↑ Bleeding time	Aslan et al. (2011a, b), Qian et al. (2012)
Rab27b−/− −Rab27a$^{ash/ash}$	Normal	Impaired dense granule number and secretion	↓↓ Dense	↓ COLL and U46			↑ Bleeding time	Tolmachova et al. (2007)

Symbols: increase—↑ mild, ↑↑ moderate, ↑↑↑ severe; decrease—↓ mild, ↓↓ moderate, ↓↓↓ severe
LD low dose agonists, *HD* high dose agonists, *3D* three-dimensional thrombi, *ADP* adenosine diphosphate, *U46* U46619: TxA2 stable analog, *THR* thrombin, *PAR4p* PAR4 agonist peptide, *CRP* collagen related peptide, *COLL* collagen, *FBN* fibrinogen, *RHOD* rhodocytin, *dense* dense granules, *alpha* alpha granules

platelets lacking the sensitivity and speed provided by CalDAG-GEFI fail to form three-dimensional thrombi particularly at arterial shear rates (Stolla et al. 2011b), and *Caldaggef1*$^{-/-}$ mice are strongly protected from ferric chloride-induced carotid artery thrombosis (Stolla et al. 2011a) and collagen- (Crittenden et al. 2004) or immune complex-induced (Amirkhosravi et al. 2014) pulmonary embolism.

Both mouse and human platelets lacking functional CalDAG-GEFI can still undergo slow but sustained RAP1 activation that supports the formation of small and stable three-dimensional thrombi at venous shear conditions (Canault et al. 2014; Stolla et al. 2011a). This CalDAG-GEFI-independent activation of RAP1 and $\alpha_{IIb}\beta_3$ is inhibited by antagonists of PKC, $P2Y_{12}$, and PI3K (Cifuni et al. 2008; Franke et al. 2000; Lova et al. 2002, 2003; Stefanini et al. 2009; Woulfe et al.

2002). PKC contributes to RAP1 activation indirectly, by stimulating granule (ADP) release, and directly by promoting delayed GTP loading of RAP1 via an unknown mechanism. The ADP receptor $P2Y_{12}$ (and probably other $G_{i/z}$-coupled receptors, see earlier) mediates a slow but sustained RAP activation by inactivating the most abundant platelet RAP-GAP, RASA3, in a PI3K-dependent manner (Stefanini et al. 2015). RASA3 is a dual-specificity GAP capable of stimulating the GTPase activity of both RAS- and RAP-GTPases depending on the cellular context (Cullen et al. 1995). It contains a unique PH/Btk domain that binds with high affinity to phosphatidylinositol 4,5-diphosphate (PIP_2) ($K_D = 0.8 \pm 0.5$ μM) and phosphatidylinositol 3,4,5-triphosphate (PIP_3) ($K_D = 0.5 \pm 0.2$ μM), the lipid product of PI3K. This interaction enables RASA3 to localize constitutively to the plasma membrane and to be dynamically regulated in response to agonist-induced PI3K signaling (Cozier et al. 2000). The only other RAP-GAP suggested to play a role in platelets is RAP1GAP2 (Schultess et al. 2005); however, RAP1GAP2 is very weakly expressed in platelets (Burkhart et al. 2012; Rowley et al. 2011; Simon et al. 2014) and genetic evidence confirming its contribution to platelet function is lacking.

While detrimental to hemostatic plug formation, RASA3 is important to maintain circulating platelets in a quiescent state. Genetic deletion (*Rasa3*$^{-/-}$; *Rasa3*$^{fl/fl}$ Pf4-Cre+) (Stefanini et al. 2015) or inactivation (*Rasa3*$^{\Delta GAP}$) (Molina-Ortiz et al. 2014) of *Rasa3* leads to severe thrombocytopenia and high embryonic/perinatal lethality due to impaired vascular development in mice. Thrombocytopenia but normal development was observed in hypomorphic mice with a single point mutation (H794L) in the C-terminal region of RASA3 (*Rasa3*^{hlb}) (Stefanini et al. 2015). Thrombocytopenia in *Rasa3* mutant mice is largely the result of platelet preactivation and impaired platelet survival in circulation (Stefanini et al. 2015), but a defect in platelet production in vitro was also reported (Molina-Ortiz et al. 2014). Interestingly, concomitant deletion of *Caldaggef1* leads to a normalization of platelet lifespan and count in *Rasa3* mutant mice. Thus, a tight regulation of the antagonistic balance between CalDAG-GEFI and RASA3 is critical for both the prevention of premature platelet activation in circulation as well as for hemostatic plug formation.

Beyond their fundamental role in integrin inside-out activation, RAP-GTPases also stimulate thromboxane A_2 generation by signaling through the MAPK ERK (Stefanini et al. 2009) and modulate the actin cytoskeleton through a complex cross-talk with RHO-GTPases (Canault et al. 2014; Stefanini et al. 2012). Platelets with impaired RAP1 signaling also show defects in integrin outside-in signaling (Bernardi et al. 2006; Zhang et al. 2011) and in granule secretion (Stefanini et al. 2012; Zhang et al. 2011). Interestingly, RAP1 was shown to bind directly to granules in platelets (Berger et al. 1994; Nagata and Nozawa 1995) and in other cell types (Maridonneau-Parini and de Gunzburg 1992; Williams et al. 2009), suggesting that it may be controlling secretion not only through the regulation of the cytoskeleton, but also more directly, for instance through the recruitment of RAL-GTPases (see "RAL Subfamily" section). Interestingly, the molecular details for how active RAP1 controls these various cellular functions, including integrin activation, are ill understood. RAP1-GTP-interacting adaptor molecule (RIAM) was suggested to mediate the interaction of RAP1 and TALIN at the β3 integrin cytoplasmic tail (Han et al. 2006; Lee et al. 2009; Watanabe et al. 2008). However, studies in *Riam* knockout mice did not confirm this hypothesis (Stritt et al. 2015; Su et al. 2015).

RAS Subfamily

Ras GTPases have a well-documented role in cell growth, differentiation, and proliferation, cellular functions that are important in megakaryocytes (Matsumura et al. 1998) but not in platelets. Consistently, RAS is expressed in very modest amounts in platelets (Burkhart et al. 2012; Ohmori et al. 1988; Rowley et al. 2011; Simon et al. 2014), where it is activated upon cellular stimulation (Shock et al. 1997). However, it is currently unclear whether RAS plays a role in platelet biology, especially as its activation is not coupled to the activation of ERK MAP kinase (Tulasne et al. 2002), a common downstream target of RAS in other cells.

RAL Subfamily

RAL regulates vectorial targeting of secretory vesicles by direct binding of the exocyst component SEC5 (Moskalenko et al. 2002). In platelets, RAL is activated in a calcium-dependent manner (Wolthuis et al. 1998), and this activation shows strong correlation with the activation of RAP1, a potential upstream regulator. RAL associates with dense granules (Mark et al. 1996) and binds SEC5 upon thrombin stimulation with the same time course as dense granule secretion (Kawato et al. 2008). Further studies need to address the interplay between different branches of the RAS superfamily (RAL, RABs, RHOs, and RAP1) that regulate exocytosis (Novick and Guo 2002) and whether RAL is downstream of RAP1.

RHEB Subfamily

In other cells RHEB is implicated in initiating mTOR (the mammalian target of rapamycin) signaling (Long et al. 2005), a pathway with documented function in platelets (Aslan et al. 2011b; Moore et al. 2014). However, RHEB is not very abundant in platelets and its contribution to cellular activation has not been studied.

RHO Family

RHO (*Ras homologous*) GTPases are best known for their role in the regulation of cytoskeletal dynamics, but they also control

multiple cell signaling pathways. Among the 20 members of the family expressed in mammalian cells, RHOA, RAC1, and CDC42 are the best characterized RHO family members in platelets. However, human platelets also express a significant amount of RHOC, RAC2, RHOG, and RHOF (RIF) (Fig. 4), which may have unique or redundant functions.

RHO

The three highly homologous RHO isoforms, RHOA, RHOB, and RHOC, when overexpressed in cells, induce the formation of stress fibers (Aspenstrom et al. 2004; Ridley and Hall 1992), structures that mediate cell retraction, an inward movement of the membranes and the associated cytoskeleton, opposed to cell spreading. In platelets, the functions of RHO GTPases have been investigated extensively using the clostridial exoenzyme C3 transferase, which modifies and blocks all three isoforms. Furthermore, two independently generated knock-out mouse models (*RhoA*$^{fl/fl}$ *Pf4-Cre*$^{+}$) that lack RHOA specifically in the megakaryocyte lineage have been described (Pleines et al. 2012; Suzuki et al. 2013). *RhoA*$^{fl/fl}$*Pf4-Cre*$^{+}$ mice display macrothrombocytopenia (50 % fewer platelets, 25 % larger volume) and more roundish shape (but normal intracellular morphology) due to aberrant megakaryocyte development and platelet production (Gao et al. 2012; Pleines et al. 2012; Shi et al. 2014; Suzuki et al. 2013). Pharmacological and genetic studies concur that RHO, by orchestrating cytoskeletal dynamics, controls three main platelet responses—shape change (Bauer et al. 1999; Pleines et al. 2012), granule secretion (Jin et al. 2009; Pleines et al. 2012), and clot retraction (Flevaris et al. 2007, 2009; Pleines et al. 2012)—while it is dispensable for integrin inside-out activation and spreading (Leng et al. 1998; Pleines et al. 2012; Schoenwaelder et al. 2002). RHO does not affect actin polymerization (Hartwig et al. 1995; Leng et al. 1998; Pleines et al. 2012), but it regulates how microtubules and actin filaments are organized (Leng et al. 1998; Pleines et al. 2012). The weakening of the cytoskeleton results in an inability to form sustained platelet–matrix interactions under flow (Schoenwaelder et al. 2002), and in smaller and less stable thrombi ex vivo and in vivo. Hence platelet-specific RHOA deficiency in mice causes a bleeding diathesis and protection from experimental thrombosis and cerebral ischemia (Pleines et al. 2012).

Dynamic control of platelet morphology and contractility is achieved through the tight regulation of RHO activity over time (Shen et al. 2013) and the cross-talk with other small GTPases, particularly with RAC1 (Flevaris et al. 2009; Gratacap et al. 2001; Shen et al. 2012) (see "RAC" section). The temporal regulation of RHO is orchestrated by the heterotrimeric G-protein subunit $G_{13\alpha}$ and integrin $\alpha_{IIb}\beta_3$ (see earlier). Stimulation of G-protein receptors coupled to $G_{13\alpha}$, such as the thrombin (PAR) and the thromboxane A_2 (TP) receptors, triggers the early RHOA signaling pathways that mediate shape change and granule secretion (Klages et al. 1999; Moers et al. 2003). Upon integrin engagement, RHOA activity is transiently downregulated in a c-SRC-dependent manner to enable RAC-dependent platelet extension and consequent spreading (early phase of outside-in signaling) (Gong et al. 2010). Once the platelets are firmly attached, CALPAIN cleaves the integrin β3 tail (Kuchay et al. 2012) and relieves c-SRC-dependent RHOA inhibition (late phase of outside-in signaling) thereby allowing platelets to contract (Flevaris et al. 2007). RHOA-powered retractile forces transmitted along the cytoskeleton–integrin–matrix axis to the underlying tissue determine clot retraction.

Very little is known about the molecular identity of the RHOA upstream regulators. p115RHOGEF (ARHGEF1) is activated by $G_{13\alpha}$ and mediates the early RHOA activation, shape change, and secretion (Hart et al. 1998; Huang et al. 2007; Kozasa et al. 1998). LARG (ARHGEF12) and GEF-H1 (ARHGEF2) are RHO-GEFs activated when integrins experience tensional forces (Guilluy et al. 2011), thus they are plausible candidates to mediate the activation of RHO during late outside-in signaling in platelets. However, *Larg*$^{-/-}$ platelets have a very modest phenotype, which seems to suggest a role of LARG downstream of G_{13}-coupled receptors, instead of the integrin (Williams et al. 2015). An important inhibitor of RHO signaling, p190RHOGAP, was not detected by platelet proteomics and transcriptomics analysis. The only RHO-GAPs characterized more in depth in platelets are OLIGOPHRENIN (ARHGAP41) (Bleijerveld et al. 2013; Elvers et al. 2012; Fotinos et al. 2015) and NADRIN (ARHGAP17, RICH1) (Beck et al. 2013; Nagy et al. 2015), but their roles are still not clear since they have broad specificity for RHOA, CDC42, and RAC1. Future studies need to address the contribution of more highly expressed RHO regulators such as p50RHOGAP/ARHGAP1, ARHGAP18, ARHGAP6, and ARAP1. RHO-GDIs are also present in very high levels in platelets (Burkhart et al. 2012; Rowley et al. 2011; Simon et al. 2014), but their role has not been investigated so far.

The best-known downstream effectors of RHO are the Rho-associated protein kinases ROCK1 and ROCK2. These serine/threonine kinases, once stimulated by RHOA/B/C-GTP, phosphorylate many substrates implicated in cell contractility and cytoskeleton remodeling, most notably myosin light chain phosphatase (MLCP) (Bauer et al. 1999; Suzuki et al. 1999) and LIM kinase (LIMK) (Dasgupta et al. 2013). Phosphorylation of MLCP inactivates its phosphatase activity and enables the increase in phosphorylation of myosin light chain (MLC), which in turn triggers myosin IIA ATPase activity and promotes the acto-myosin contractile

response that is involved in platelet shape change, clot retraction, and secretion. Conversely, LIMK, when phosphorylated, is able to inhibit (by phosphorylation) the actin-depolymerizing activity of COFILIN, leading to stabilization of filamentous actin structures, decreased branching, and inhibition of cell extension. Inhibition of both ROCK isoforms with pharmacological inhibitors recapitulates many features of *RhoA*-deficient platelets (Bauer et al. 1999; Calaminus et al. 2007a; Schoenwaelder et al. 2002; Suzuki et al. 1999). However, *Rock1*-deficiency impairs LIMK/COFILIN phosphorylation but not MLCP phosphorylation and shape change (Dasgupta et al. 2013), suggesting that the two isoforms (ROCK1 and ROCK2) may have different functions and that the RHO-dependent contractile response may be under the control of the more abundant isoform ROCK2.

Formins, a large class of multidomain proteins that control both actin polymerization and microtubule stabilization, are another important class of RHO effectors (Faix and Grosse 2006). Platelets have been shown to express mDIA1, DAAM (Higashi et al. 2008), and FHOD1 (Thomas et al. 2011); however, $mDia^{-/-}$ platelets display no functional defects (Thomas et al. 2011).

RAC

RAC GTPases, when overexpressed in cells, stimulate the extension of lamellipodia and cell spreading, an outward movement of the membranes and the underlying cytoskeleton (Aspenstrom et al. 2004; Ridley et al. 1992). Proteomics and transcriptomics studies (Burkhart et al. 2012; Rowley et al. 2011; Simon et al. 2014) suggest that platelets express two of the known RAC isoforms, RAC1 and RAC2, which have both unique and overlapping roles in other hematopoietic cells. The role of RAC2 in platelets has been disregarded because it could not be detected by immunoblotting and its deletion in mouse platelets was inconsequential (McCarty et al. 2005); however, activation of human platelets may depend more on this RAC isoform as they express more RAC2 than murine cells (Burkhart et al. 2012; Zeiler et al. 2014). Germline deletion of *Rac1* is lethal in mice, thus RAC1 functions in platelets have been investigated with inducible ($Rac1^{fl/fl}$ *Mx-Cre*$^{+}$) (Akbar et al. 2007; McCarty et al. 2005) or conditional ($Rac1^{fl/fl}$ *Pf4-Cre*$^{+}$) (Delaney et al. 2012; Pleines et al. 2009) knockout mouse models and two small-molecule inhibitors, NSC23766 (Akbar et al. 2007) and EHT1864 (Pollitt et al. 2010; Stefanini et al. 2012). It is important to note, however, that the specificity of these compounds has recently been questioned (Dutting et al. 2015).

These studies demonstrated that RAC1 controls both cytoskeleton-dependent and cytoskeleton-independent platelet responses, and that *Rac1*-deficient platelets lack the ability to form three-dimensional thrombi under flow ex vivo and in vivo (McCarty et al. 2005). Consistently, *Rac1* mutant mice are protected from thrombosis (McCarty et al. 2005; Pleines et al. 2009) and have prolonged bleeding times (Akbar et al. 2007).

The most notable non-cytoskeletal function of RAC1 is the regulation of PLCγ_2 activity (Piechulek et al. 2005). Platelets lacking functional RAC1 exhibit normal phosphorylation of PLCγ_2, but reduced production of IP_3, impaired intracellular calcium mobilization (Pleines et al. 2009), and impaired PKC activation (Guidetti et al. 2009). Consequently, in the absence of RAC1 the major signaling defect is downstream of ITAM-coupled receptors (Pleines et al. 2009) and integrins (Guidetti et al. 2009), which signal to PLCγ_2. In addition, RAC1 is implicated in the initial signaling steps following GPIb-IX-V stimulation, linking the Src family kinase LYN to MAPK and PI3K/AKT signaling (Delaney et al. 2012).

Besides its role in signaling, RAC1 is best known for orchestrating cytoskeletal dynamics and for inducing cell spreading. Deletion or inhibition of RAC1 in mouse platelets reduces actin polymerization (Hartwig et al. 1995; McCarty et al. 2005), prevents the formation of lamellipodia without interfering with filopodia extension (McCarty et al. 2005), partially impairs both α- and dense-granule secretion (Akbar et al. 2007), and completely inhibits clot retraction (Flevaris et al. 2009) without affecting platelet shape change (McCarty et al. 2005).

It is well established that RAC1 mediates lamellipodia formation through the activation of ARP2/3, a protein complex that nucleates the growth of a new actin filament on the side of an existing filament and, as a result, creates branched actin networks. In platelets, RAC1 and ARP2/3 are enriched in the actin nodules during early spreading (Calaminus et al. 2008; Poulter et al. 2015), and later colocalize at the edge of the fully extended lamellipodia (McCarty et al. 2005). The effectors that link RAC1-GTP to ARP2/3 activation are members of the SCAR/WAVE family. Deficiency of SCAR/WAVE-1 in mice impairs lamellipodia formation on CRP and laminin, but not on collagen or fibrinogen-coated surfaces (Calaminus et al. 2007b). This incomplete spreading defect may be explained by redundant signaling by SCAR-WAVE-2, which is also abundant in platelets. Other major RAC1 (and CDC42) effectors are the serine–threonine kinases of the PAK family. In platelets, RAC1 employs the PAK signaling system to initiate a number of parallel pathways. Pharmacological inhibition of PAKs reduces the phosphorylation of GIT, GEFH1, LIMK, MERLIN, MEK, ERK, AKT, GSK3β (Aslan et al. 2013a, 2013b) and impairs multiple RAC1-dependent platelet responses.

RAC1 orchestrates cytoskeletal dynamics and promotes platelet activation through a complex cross-talk with RHOA and RAP1. The functional relationship with RHOA varies over time (Shen et al. 2012). Upon platelet stimulation,

RHOA-mediated shape change is dependent on G_{13} signaling, while RAC1 activation depends on G_q or ITAM signaling and is required for actin polymerization (Gratacap et al. 2001). Both RHOA and RAC1 positively regulate granule secretion (Flevaris et al. 2009) and generation of PIP_2 (Chatah and Abrams 2001; Gratacap et al. 2001). Upon integrin engagement, during the early phase of outside-in signaling, RAC1 and RHOA have antagonistic functions. RAC1 stimulates actin polymerization, F-actin branching, and lamellipodia extension, while RHOA is turned off to prevent cell retraction and allow spreading (Gong et al. 2010; Pleines et al. 2012). In the final stage of outside-in signaling, after calpain has cleaved the integrin β3 tail, RAC1 and RHOA are both active and have synergistic functions that converge at the level of MLC phosphorylation and lead to platelet-dependent clot retraction. RAC1 increases MLC phosphorylation through the stimulation of the MAPKs ERK and p38 and the activation of myosin light chain kinase (MLCK), while RHOA acts through its effector ROCK to phosphorylate and inhibit MLCP (Egot et al. 2013; Flevaris et al. 2009). Concurrently, there is cross-talk between RAC1 and RAP1, which is critical for full platelet activation (Stefanini et al. 2012). RAC1 promotes RAP1 activation indirectly by stimulating calcium mobilization and ADP secretion, which mediate CalDAG-GEFI activation and RASA3 inhibition, respectively (see earlier). In turn, RAP1-GTP promotes sustained RAC1 activation, which is critical for lamellipodia extension (Canault et al. 2014; Stefanini et al. 2012).

The best characterized RAC-GEFs expressed in platelets are VAV1 and VAV3, which play a crucial and redundant role in the regulation of spreading and in the activation of $PLC\gamma_2$ following stimulation of ITAM-coupled receptors (Pearce et al. 2004; Suzuki-Inoue et al. 2006) or integrin $\alpha_{IIb}\beta_3$ (Pearce et al. 2007). However, VAVs are known to have a broad specificity and the phenotype of *Vav1*/*Vav3*-double knockouts is similar but not identical to the phenotype of *Rac1*-knockouts, suggesting that VAV1/3 may also be modulating other RHO-GTPases. Other potential RAC-GEFs were identified in platelets (Aslan et al. 2011a, 2013a). Among these, P-REX1 does not seem to be a major player since its deficiency in mouse platelets has no effect on spreading (Aslan et al. 2011a) and has a modest effect on aggregation and secretion stimulated by low, but not high, doses of thrombin and U46619 (Qian et al. 2012). β-PIX (ARHGEF7, COOL-1), the most abundant RHO-GEF detected in platelets, and α-PIX (ARHGEF6, COOL-2) could be regulating integrin-induced RAC1 activation (ten Klooster et al. 2006), since they bind GIT1 (Nagy et al. 2015; Sato et al. 2008), an ARF-GAP that translocates to the cytoskeleton upon integrin engagement, but a detailed analysis of their functional importance in platelets is still lacking. TIAM has also been implicated in integrin-dependent RAC1 activation, downstream of a kinase cascade involving SRC, SYK, mTOR kinase, and the ribosome protein S6 kinase (S6K1) (Aslan et al. 2011b); however, this GEF also awaits further investigation. Finally, in addition to the canonical RAC-GEFs, platelets also express several DOCK proteins, which can act as powerful GEFs for RAC and CDC42 (Gadea and Blangy 2014).

RHOG

RHOG shares ~70 % sequence homology with RAC GTPases (Vincent et al. 1992) and, just like these closely related small GTPases, its ectopic expression in cell lines induces lamellipodia formation (Aspenstrom et al. 2004). In platelets, RHOG is rapidly activated in response to either G protein or ITAM-coupled receptor stimulation. However, its deficiency does not affect spreading, clot retraction, and $PLC\gamma_2$ activation, possibly because of redundancy with RAC1. The only defects of $RhoG^{-/-}$ platelets are a decrease in α- and dense-granule secretion and a reduction of SYK, ERK, and AKT phosphorylation, solely downstream of the collagen receptor GPVI. This signaling impairment results in a modest aggregation defect in vitro, which can be rescued by addition of ADP, and reduced thrombus formation but normal hemostasis in vivo (Goggs et al. 2013a; Kim et al. 2013b).

CDC42

In mammalian cells, CDC42 is important for the formation of actively protruding filopodia (Nobes and Hall 1995), the regulation of microtubule dynamics, cell polarity (Etienne-Manneville 2004), cell contraction (Wilkinson et al. 2005), and membrane trafficking (Kroschewski et al. 1999; Osmani et al. 2010). In human platelets, CDC42 is robustly activated in response to thrombin stimulation (Dash et al. 1995) or upon platelet adhesion to collagen (Pula and Poole 2008), but its intracellular distribution and its sensitivity to agonists is distinct from that of RAC1 (Vidal et al. 2002). Moreover, CDC42 is the specific activator of the Wiskott–Aldrich Syndrome protein (WASP), a major regulator of actin polymerization (Pollitt and Insall 2009; Rohatgi et al. 1999) that also binds microtubules (Tian et al. 2000). The role of CDC42 in platelet function was assessed in $Cdc42^{fl/fl}$*Pf4-Cre*+ (Pleines et al. 2010) and $Cdc42^{fl/fl}$*Mx-Cre*+ mice (Akbar et al. 2011). Both mouse models are characterized by a partial macrothrombocytopenia (platelet count 50–80 % of controls) and a mildly prolonged bleeding time. However, the underlying phenotypes are conflicting. Constitutive deletion of the *Cdc42* gene in megakaryocytes ($Cdc42^{fl/fl}$*Pf4-Cre*+) results in platelets with no defect in filopodia formation in suspension or upon adhesion to fibrinogen, in either unstimulated or stimulated conditions.

Reduced filopodia formation is only observed upon platelet adhesion to VWF, suggesting that CDC42 might have a specific role downstream of the GPIb-IX-V receptor. Unexpectedly, these platelets exhibit an increased ATP content and increased secretion of both α- and dense-granules, enhanced aggregation in vitro, accelerated thrombus formation under flow, shorter occlusion time in vivo, and a slightly shorter half-life, suggesting that CDC42 controls granule packing and release (Pleines et al. 2010). Conversely the inducible deletion of the *Cdc42* gene in the hematopoietic system (*Cdc42*$^{fl/fl}$*Mx-Cre+*) leads to reduced filopodia formation on immobilized fibrinogen or collagen-related peptide, an impaired secretion and aggregation response, and diminished phosphorylation of RAC/CDC42 downstream effectors such as PAK1/2 (Akbar et al. 2011). The reasons for these divergent results are currently unknown. Moreover, circulating platelets in *Rac1*$^{fl/fl}$*Cdc42*$^{fl/fl}$ *Pf4-Cre+* mice completely lack microtubule coils in the marginal band and show a dysregulation in granule numbers (Pleines et al. 2013), suggesting that CDC42 might also contribute to granule packing and microtubule organization in the developing megakaryocyte.

RHOF

Recent data suggest the existence of CDC42-independent mechanisms of filopodia formation, involving, among others, the Rho GTPase RHOF (RIF) (Ellis and Mellor 2000). However, *RhoF*$^{-/-}$ mice display no alterations in their platelets and megakaryocytes (Goggs et al. 2013b). Consistent with this, deficiency of the non-branching actin nucleating agent mDIA, which is known to bind RHOF, also has no effect on platelet activation and morphology (Thomas et al. 2011).

RAB Family

RAB (*Ras-related proteins in brain*) GTPases are master regulators of vesicular/membrane trafficking (Stenmark 2009). Thus far 70 members of the RAB family have been identified in humans and platelets express about 40 of them (Fig. 4). There is additional complexity to this family as each RAB protein is suggested to be enriched in a specific membrane microdomain (Chavrier et al. 1990), where it can regulate distinct steps during vesicular/membrane trafficking. These include cargo selection, vesicle budding from a donor membrane, vesicle transport along cytoskeletal tracts (microtubules or actin-filaments), or tethering/fusion to the correct acceptor membrane. Importantly, RABs are also indirectly involved in signal transduction by regulating the exposure/internalization of surface receptors and the translocation of signaling molecules between intracellular compartments.

Proper post-translational modification and localization of RABs is essential for optimal platelet production and function. Mice homozygous for *gunmetal*, a spontaneous, recessive mutation in the *Rab geranylgeranyl transferase* α subunit gene (*Rabggta*), display macrothrombocytopenia, reduced platelet α- and dense-granule contents, and prolonged bleeding times due to the hypo-prenylation of several platelet RABs and their consequent displacement in the cytosolic fraction (Detter et al. 2000; Novak et al. 1995; Swank et al. 1993).

In platelets, the best-studied members of this family are RAB27B and RAB27A, which regulate specifically dense granule number and secretion (Tolmachova et al. 2007). *Rab27b*-deficient platelets exhibit a 50 % reduction in dense granule number and content and a severe, but incomplete, defect in dense granule secretion. As a consequence, these mice display a reduced aggregation response to collagen and U46619 and a bleeding defect that is more severe than that of the gunmetal mouse model (Tolmachova et al. 2007). RAB27A is less abundant than RAB27B in platelets. Its deficiency in humans causes type 2 Griscelli syndrome, a rare autosomal recessive disorder characterized by hypopigmentation and immunodeficiency due to a defect in the release of melanosomes of melanocytes and secretory lysosomes of lymphocytes, organelles that are closely related to platelet dense granules. The mouse model of this disease, a spontaneous *Rab27a* knockout line designated *ashen*, on some genetic backgrounds (on C3H/HeSnJ, but not on C57BL/6J) displays a marginal defect in platelet dense granule secretion and a bleeding tendency (Novak et al. 2002; Wilson et al. 2000). Studies with double-deficient mice (*Rab27b*$^{-/-}$*Rab27a*$^{ash/ash}$) suggest that the two isoforms could be to a certain extent redundant since RAB27A can partially compensate for the secretory defect but not for the reduced granule number of *Rab27b*-deficient platelets (Tolmachova et al. 2007). They also share effectors such as Munc13-4 (Shirakawa et al. 2004), a critical component of the platelet secretory apparatus (Ren et al. 2008). Besides RAB27B, RAB38 has been implicated in dense granule biogenesis (Ambrosio et al. 2012; Ninkovic et al. 2008).

Very little is known about the function and localization of the numerous other platelet RABs and about how they regulate vesicle traffic in platelets. Adding to the complexity, α-granules are ten times more abundant than dense granules and several studies suggest the existence of heterogenous subpopulations, which may be regulated by distinct RAB isoforms. A study dating back to the early '90s has shown that RAB6 and RAB8 are preferentially targeted to the α-granules (Karniguian et al. 1993). More recently, a permeabilized platelet system was employed to show that a RAB-GDI binding RAB4, RAB5, RAB6, and RAB8, but not RAB27, inhibits α- but not dense-granule secretion and RAB4 positively regulates Ca^{2+}-induced secretion of α-granules (Shirakawa et al. 2000). However, more detailed studies are needed to dissect the role of the distinct members of the RAB family in the platelet-specific membrane traffic pathways.

ARF Family

ARF (*ADP ribosylation factor*) GTPases are the founding members of a growing family that includes ARL (*Arf-like*), ARP (*Arf-related proteins*), and the remotely related SAR (*Secretion-associated and Ras-related*) proteins. Members of this family are known to regulate membrane/vesicular trafficking through (1) membrane recruitment of effector proteins that orchestrate vesicle budding; (2) stimulation of enzymes that alter membrane lipid composition, such as phospholipase D and phosphatidylinositol-4-phosphate-5-kinase; and (3) modulation of RHO-GTPases, which in turn regulate the cytoskeleton. Platelets express several members of the ARF family (Fig. 4), but the only member of the family that has been studied in detail is ARF6, which is active (GTP-bound) in resting platelets and rapidly down-regulated upon platelet stimulation with either collagen or thrombin (Choi et al. 2006; Karim et al. 2008). Using a cell-permeant myristoylated peptide that interferes with ARF6-GTP hydrolysis, it was shown that ARF6 down-regulation is critical to enable agonist-induced RHOA, RAC1, and CDC42 (but not RALA or RAP1) activation and the RHO-dependent functional responses required for optimal platelet activation (Choi et al. 2006). ARF6 deficiency in mice interferes with the uptake of fibrinogen through integrin $\alpha_{IIb}\beta_3$, which results in enhanced clot retraction and spreading (Huang et al. 2016). It has also been shown that the ARF-GAP GIT1 constitutively binds the RAC-GEFs, α-PIX (ARHGEF6, COOL-2), and β-PIX (ARHGEF12, COOL-1), and that this interaction is important for their recruitment to the cytoskeleton upon integrin engagement in platelets (Nagy et al. 2015; Sato et al. 2008). The mechanistic basis of the RHO/ARF cross-talk in platelets needs to be elucidated further and a possible avenue of investigation is the study of ARF-GEFs and/or ARF-GAPs with multiple functional domains such as ARAP1, a PIP_3-dependent ARF-GAP that also includes a RHO-GAP domain (Miura et al. 2002).

RAN Family

The RAN (*Ras-like nuclear*) GTPase is the only member of the RAN family in humans. In nucleated cells it is best known for regulating the nucleocytoplasmic traffic of RNA and proteins, but it also orchestrates mitotic spindle assembly by controlling microtubule organization (Li et al. 2003).

In platelets, RAN is quite abundant despite the lack of nuclei (Fig. 4). Deletion of the RAN-binding protein RANBP10 in mice leads to a prolonged bleeding time (Kunert et al. 2009), formation of unstable thrombi in an arterial thrombosis model, attenuated platelet shape change, reduced secretion and aggregation, and impaired marginal band contraction during platelet activation (Meyer et al. 2012), suggesting that RAN in platelets could be regulating microtubule dynamics.

Concluding Remarks

GTPases play a central role in the intracellular signaling required for platelet function. Heterotrimeric G proteins transmit signals from GPCRs, which control both platelet inhibition in circulation and platelet activation at sites of mechanical injury. G_q proteins are critical for the rapid generation of Ca^{2+} and DAG, second messengers that facilitate the near-immediate inside-out activation of integrin receptors on the cell surface and the release of autocrine/paracrine agonists. Proteins of the G_i family are critical to sustain platelet activation and integrin-mediated adhesion. G_{13} proteins are important to facilitate shape change and clot retraction. G_s proteins antagonize the above-mentioned processes by activating PKA, a kinase that down-modulates various signaling pathways. Small GTPases play a crucial role in the integration of stimulatory and inhibitory signals provided by heterotrimeric G proteins. RAP GTPases are critical for integrin activation downstream of G_q and G_i signaling. RHO GTPases are master regulators of the cytoskeleton and thus control shape change, spreading, and clot retraction downstream of G_q and G_{13} signaling. Together with RAB and ARF GTPases, they also control granule secretion. As we learn more about these systems, it is becoming increasingly apparent that (1) signaling by heterotrimeric and small GTPases is very interconnected, and (2) both bleeding and thrombotic complications can often be traced back to disturbances in GTPase signaling. Given their central role in platelet activation, it is also not surprising that antiplatelet therapies used in the clinic target GTPase signaling (Gurbel et al. 2015; Jackson and Schoenwaelder 2003; Smyth et al. 2009). Thus, a better understanding of the unique contributions to platelet activation of the various members of this vast family will be key to the development of improved diagnostics and therapies for cardiovascular disease.

Take Home Messages

- Heterotrimeric G proteins and small GTPases are molecular switches that cycle between the inactive, GDP-bound and the active, GTP-bound state. The GDP/GTP cycle is controlled by GEFs and GAPs.
- Heterotrimeric G proteins are critical for the function of G protein-coupled receptors (GPCRs) that respond to soluble agonists such as thrombin (PAR1, PAR4), ADP (P2Y1, P2Y12), and thromboxane A_2 (TP).
- Platelets utilize G_q, $G_{12/13}$, G_i, and G_s proteins to fine-tune their activation state at sites of vascular injury.

(continued)

- Small GTPases play a crucial role in the integration of stimulatory and inhibitory signals provided by GPCRs.
- The best studied small GTPases in platelets are members of the RAS, RHO, and RAB families, which play crucial roles in integrin signaling (adhesion), cytoskeletal dynamics (shape change, clot retraction), and vesicular trafficking (granule secretion), respectively.
- Interference with GPCR and small GTPase signaling is critical to current and emerging antiplatelet therapies.

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Platelet Signalling: Calcium

Alan G.S. Harper and Stewart O. Sage

Abstract

A rise in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) is central to platelet activation. Agonists stimulate a rise in $[Ca^{2+}]_{cyt}$ through a combination of Ca^{2+} release from intracellular stores located in the dense tubular system (DTS) and acidic organelles as well as Ca^{2+} entry across the plasma membrane via several channel types. $[Ca^{2+}]_{cyt}$ may be reduced by Ca^{2+} sequestration into the intracellular stores by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and via a H^+-dependent mechanism, whilst Ca^{2+} may be removed across the plasma membrane by plasma membrane Ca^{2+}-ATPases (PMCAs) and by Na^+/Ca^{2+} exchangers (NCXs). Ca^{2+} signals are shaped by differential employment of these basic Ca^{2+} entry and removal processes and by Ca^{2+} buffers present in the platelet cytosol and other cellular compartments. In turn, Ca^{2+} signals can be transduced into a number of platelet responses by an array of effector proteins which may be activated in some cases by Ca^{2+} signals confined to specific cellular microdomains.

Introduction

Human platelets maintain a low resting cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) estimated to be around 50–100 nM. To maintain the resting $[Ca^{2+}]_{cyt}$ against leakage of Ca^{2+} from intracellular stores or across the plasma membrane, or to restore resting $[Ca^{2+}]_{cyt}$ after the generation of Ca^{2+} signals, several Ca^{2+} removal mechanisms are used. Ca^{2+} is sequestered into organelles by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and by a vacuolar H^+-ATPase (vH^+-ATPase), probably coupled to a H^+/Ca^{2+} exchanger. Ca^{2+} is removed across the plasma membrane either by primary active transport via plasma membrane Ca^{2+}-ATPases (PMCAs) and by Na^+/Ca^{2+} exchangers (Fig. 1).

Platelet activation is associated with a rise in $[Ca^{2+}]_{cyt}$, which may reach values of several μM and be associated with oscillations or repetitive spikes in elevated $[Ca^{2+}]_{cyt}$ in individual cells. Stored Ca^{2+} may be released from the dense tubular system and acidic organelles by the second messengers inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively, and the entry of Ca^{2+} across the plasma membrane may be activated in response to store depletion (store-operated Ca^{2+} entry; SOCE), in response to the second messenger diacylglycerol (DAG) or by direct activation of an ionotropic receptor in the case of ATP acting at the P2X1 receptor. The calcium signals generated by different platelet agonists are shaped not just by the calcium signalling elements recruited but also by the Ca^{2+} buffers in the platelet cytosol.

Many of the basic aspects of platelet calcium signalling have been appreciated for a decade or more. However, many studies have relied on measuring $[Ca^{2+}]_{cyt}$ alone. Such studies are limited by their ability to only monitor the net effect

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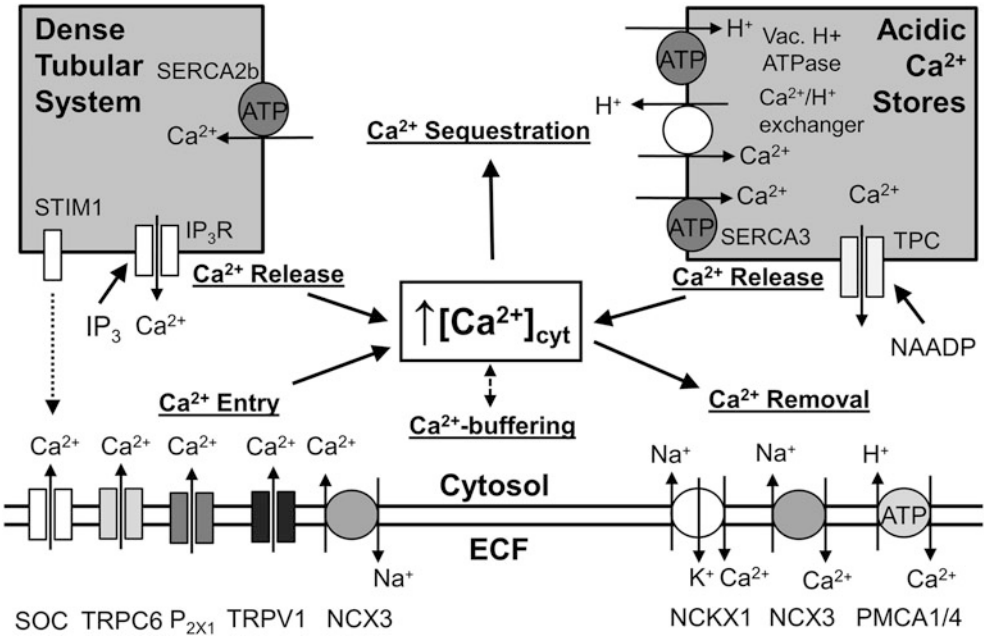

Fig. 1 Summary of the mechanisms that generate and shape platelet Ca^{2+} signals. Ca^{2+} enters the platelet cytosol by entry across the plasma membrane via several channels and possibly by reverse-mode Na^+/Ca^{2+} exchange, as well as by release from intracellular stores and acidic organelles. The Ca^{2+} signal is shaped by various cytosolic Ca^{2+} buffers. Ca^{2+} is removed across the plasma membrane by plasma membrane Ca^{2+} ATPases and forward mode Na^+/Ca^{2+} exchange, and Ca^{2+} is sequestered back into the DTS by SERCA2b and into acidic organelles by SERCA3 and Ca^{2+}/H^+ exchange powered by a V-type H^+-ATPase

of experimental manipulations on the combined actions of the component processes involved in controlling $[Ca^{2+}]_{cyt}$ (Ca^{2+} buffering, sequestration, release, entry and removal) and lack the ability to resolve the source of the Ca^{2+} or the transporters or channels affected. More recent work in human platelets has highlighted the many pitfalls of drawing conclusions as to the molecular pathways involved in eliciting Ca^{2+} signals based solely on measurements of $[Ca^{2+}]_{cyt}$ rather than methods to measure agonist-evoked changes in extracellular and intracellular store Ca^{2+} concentrations ($[Ca^{2+}]_{ext}$ and $[Ca^{2+}]_{st}$, respectively) as well as the platelet pericellular Ca^{2+} concentration ($[Ca^{2+}]_{peri}$), which can be utilised alongside the measurement of agonist-evoked changes in $[Ca^{2+}]_{cyt}$ to improve the interpretation of results compared with simple cytosolic Ca^{2+} measurements alone. Here we summarise current information on platelet calcium signalling, paying particular attention to more recent findings.

Processes That Increase Platelet Cytosolic Calcium Concentration

Release of Stored Ca^{2+}

Most platelet agonists evoke the release of Ca^{2+} from intracellular stores. Two stores are involved, the dense tubular system (DTS; or endoplasmic reticulum (ER)) and acidic stores found in lysosomes and dense granules.

The DTS represents the largest releasable Ca^{2+} store in platelets. DTS Ca^{2+} is released following the formation of inositol 1,4,5-trisphosphate (IP_3). Many agonists, including ADP, platelet activating factor (PAF), thrombin and thromboxane A_2 increase platelet IP_3 levels (Rink and Sage 1990). These agonists activate metabotropic receptors coupled to phospholipase C (PLC) β isoforms 1–3 via a heterotrimeric GTP-binding protein, Gq (Heemskerk and Sage 1994; Boyanova et al. 2012). Additionally, collagen and the low affinity antibody Fc receptor, FcγRII, activate PLC-γ2 via tyrosine phosphorylation (Daniel et al. 1994; Blake et al. 1994). Some studies have implicated PLC-γ_1 in thrombin-evoked responses, although recent data suggest this PLC isoform is undetectable in the platelet proteome (Boyanova et al. 2012). IP_3 releases stored Ca^{2+} in human platelets (O'Rourke et al. 1985) and platelets express all three isoforms of the IP_3-receptor (IP_3RI-III), with the Type I and II receptors being expressed in internal membranes and therefore apparently responsible for IP_3-evoked Ca^{2+} release (Quinton and Dean 1996; El-Daher et al. 2000).

The releasable acidic Ca^{2+} store in platelets is smaller than that in the DTS. The acidic store is released by thrombin acting via protease activated receptor-4 (PAR-4) and glycoprotein (GP) Ib-IX-V, but ADP and arginine vasopressin are apparently without effect on this store (Rosado 2011). The second messenger responsible for releasing Ca^{2+} from acidic stores is nicotinic acid adenine dinucleotide phosphate (NAADP) and this messenger has been shown to release stored Ca^{2+} in permeabilised platelets (López et al. 2006). The collagen receptor GPVI has been reported to elevate NAADP in human platelets and to release Ca^{2+} from the acidic store (Coxon et al. 2012). The identity of the NAADP receptor is a matter of ongoing debate. Much attention has focussed on two-pore channels (TPCs) although conflicting evidence exists (Morgan et al. 2015). Recent work on

platelet dense granules isolated from a megakaryocytic cell line suggests that TPC2 is present in the dense granule membrane and can mediate Ca^{2+} release from this acidic organelle (Ambrosio et al. 2015). Although the agonist-releasable Ca^{2+} pool in the acidic stores is relatively small, it may play an important role in platelet Ca^{2+} signalling by acting to sensitise IP_3Rs and so promote Ca^{2+} release from the DTS, as proposed in the trigger hypothesis demonstrated in other cell types (Galione 2015).

A third known Ca^{2+}-releasing second messenger, cyclic ADP-ribose (cADPR), has been reported to be formed in platelets in response to thrombin but this messenger is without Ca^{2+}-releasing effect in human platelets (Ohlmann et al. 1998).

Ca^{2+} Entry Across the Plasma Membrane

Plasma membrane Ca^{2+} entry channels may be receptor-, second messenger- or store-operated (Sage 1997). In addition, Ca^{2+} may enter cells by Na^+/Ca^{2+} exchangers operating in reverse mode.

Receptor-Operated Channels The only receptor-operated channel identified in human platelets is P2X1 (MacKenzie et al. 1996). This ionotropic receptor is stimulated by ATP and is non-selective, allowing Na^+ as well as Ca^{2+} across the plasma membrane (Sage et al. 1991). Earlier work suggested that this receptor was stimulated by ADP, but it was later shown that the stimulus was the contaminating ATP present in commercial ADP preparations (Mahaut-Smith et al. 2000). The presence of this receptor-operated channel was first suggested on the basis of elevations in $[Ca^{2+}]_{cyt}$ that commenced without discernible latency upon stimulation (Sage and Rink 1987) and was later confirmed in the first patch-clamp recordings from stimulated platelets (Mahaut-Smith et al. 1990). Although P2X1 receptors desensitise rapidly at high agonist concentrations they may be active for tens of seconds at lower levels of stimulation allowing P2X1 receptor stimulation to sustain Ca^{2+} signals evoked by other platelet agonists that stimulate ATP secretion from dense granules (Mahaut-Smith et al. 2011). Ca^{2+} and Na^+ entry via P2X1 receptors also results in membrane depolarisation which may enhance signalling through Gq-coupled receptors such as P2Y1 (Mahaut-Smith et al. 2011). The availability of selective agonists such as α-β-methylene ATP has demonstrated that the rises in $[Ca^{2+}]_{cyt}$ evoked by P2X1 stimulation can result in functional responses including shape change and low levels of $\alpha_{IIb}\beta_3$ activation leading to reversible aggregation, whilst transgenic mouse models indicate a role for P2X1 receptors in amplifying thrombus development (Mahaut-Smith et al. 2011). Although the P2X1 receptor is selective for ATP, it was suggested that a splice variant of this receptor, P2X1del, might mediate P2X1-like responses to ADP (Greco et al. 2001). However, electrophysiological studies indicate that P2X1del does not form functional ion channels and furthermore P2X1del protein is reported to be below the level of detection in human platelets (Vial et al. 2003).

Second Messenger-Operated Channels Second messenger-operated channels (SMOCs) are activated indirectly following the stimulation of metabotropic receptors. The leading candidates for SMOCs in human platelets are channels formed by one or more members of the transient receptor potential canonical sub-family (TRPCs). Human platelets are varyingly reported to express TRPCs 1, 3, 4, 5, 6 and 7 (Hassock et al. 2002; Brownlow and Sage 2005; Liu et al. 2008; Boyanova et al. 2012), with possible associations between TRPCs 1, 4 and 5 and between TRPCs 3 and 6 (Brownlow and Sage 2005). There is conflicting evidence regarding the gating mechanisms of the various TRPC isoforms (Hardie 2007) and at least with TRPC3 the gating mechanism may depend on expression level (Vasquez et al. 2003). TRPCs 3, 6 and 7 are generally regarded as activated by diacylglycerol (DAG; Hardie 2007). In human platelets TRPCs 3 and 6 appear to be responsible for Ca^{2+} entry downstream of DAG formation by PLCs (Hassock et al. 2002; Harper et al. 2013). TRPC6 is reported to be absent from the plasma membrane of resting human platelets and to be inserted upon stimulation by the DAG analogue, OAG, or by thrombin (Harper et al. 2013). Since TRPCs form non-selective cation channels, they gate a substantial Na^+ entry which may result in secondary Ca^{2+} entry by Na^+/Ca^{2+} exchangers (NCXs) operating in reverse mode (Harper et al. 2013).

Another potential SMOC in human platelets may be formed by transient receptor potential vanilloid-1 (TRPV1). TRPV1 has been reported to be expressed in human (Harper et al. 2009; Savini et al. 2010) but not murine platelets (Sage et al. 2014). The TRPV1 agonist, capsaicin, elevates $[Ca^{2+}]_{cyt}$ in human platelets, a response that is inhibited by the TRPV1 antagonists 5′-iodoresiniferatoxin or AMG9810 (Harper et al. 2009). These antagonists reduce rises in $[Ca^{2+}]_{cyt}$ evoked by ADP and thrombin, suggesting a contribution from TRPV1 activation, however the endogenous activator of TRPV1 in human platelets remains to be identified (Harper et al. 2009). Potential candidates include several endovanilloids (Harper et al. 2009), including 12-HPETE, which is produced upon platelet activation (Coffey et al. 2004).

Store-Operated Calcium Entry The phenomenon of store-operated Ca^{2+} entry (SOCE), where depletion of intracellular Ca^{2+} stores leads to the activation of plasma membrane channels permeable to Ca^{2+}, was first described by Putney in 1986. A kinetic study of ADP-evoked rises in $[Ca^{2+}]_{cyt}$ suggested SOCE operated in human platelets since divalent

cation entry was activated temporally coincident with store release (Sage et al. 1990). The existence of SOCE in platelets was confirmed by the use of SERCA inhibitors to deplete intracellular Ca^{2+} stores in the absence of agonist stimulation (Sargeant et al. 1992). In platelets as in other cells the quest to identify the store-operated channel (SOC) proved lengthy. There is now consensus that a highly Ca^{2+}-selective channel (under physiological conditions) is formed by Orai1 and that the Ca^{2+} sensor in the membrane of the endoplasmic reticulum responsible for its activation is STIM1 (Feske et al. 2006; Roos et al. 2005). STIM1–Orai1 coupling has been demonstrated in human platelets (Jardin et al. 2008). Although an essential role for Orai1 in SOCE and thrombus formation has been demonstrated in murine platelets in knock-out studies (Braun et al. 2009), humans with Orai1 mutations affecting channel function do not suffer from major platelet functional defects (Feske 2010).

TRPC1 has also been suggested to play a role in SOCE in human platelets (Rosado et al. 2002), although this does not appear to be the case in murine platelets (Varga-Szabo et al. 2008). There is good evidence to support a role for TRPC1 in SOCE in many other cell types (Cheng et al. 2013). As indicated earlier, TRPCs form non-selective cation channels that are permeable to Na^+ as well as Ca^{2+}. In human platelets, Ca^{2+} store depletion following SERCA inhibition elevates the cytosolic Na^+ concentration, an event blocked by an anti-TRPC1 antibody directed to the pore-forming region of the protein (Harper and Sage 2007). The same antibody reduces the rise in $[Ca^{2+}]_{cyt}$ when Ca^{2+} is added to platelet suspensions following store depletion by SERCA inhibition (Rosado et al. 2002). Although a role for TRPC1 in SOCE in human platelets remains controversial, the presence of this protein in the human platelet proteome allows for this possibility (Boyanova et al. 2012). Although it was suggested that TRPC1 might be activated by conformational coupling to the type II IP_3R, it now appears such coupling is not essential for activation (Harper and Sage 2007). TRPC1 is reported to couple to STIM1 upon Ca^{2+} store depletion in human platelets (Jardin et al. 2008), and this likely serves to activate the channel as reported in other cell types (Cheng et al. 2013).

Processes That Decrease Platelet Cytosolic Calcium Concentration

Ca^{2+} Removal Across the Plasma Membrane

Ca^{2+} is removed across the platelet plasma membrane either by primary active transport via a plasma membrane Ca^{2+}-ATPase (PMCA) or by secondary active transport via Na^+/Ca^{2+} exchangers (NCXs). PMCAs are high affinity Ca^{2+} transporters but have relatively low turnover rates, whilst NCXs have a lower Ca^{2+} affinity but higher turnover rates than PMCAs (Blaustein and Lederer 1999). Consequently, PMCAs are important in the maintenance of resting $[Ca^{2+}]_{cyt}$ whilst Na^+/Ca^{2+} exchangers are more important in the restoration of $[Ca^{2+}]_{cyt}$ after it has been elevated during Ca^{2+} signalling.

Of the four isoforms on PMCA, only PMCA1b and PMCA4b have been detected in human platelets (Martin et al. 2000). PMCA1b expression in human platelets is very low (Pászty et al. 1998), suggesting PMCA4b is the dominant isoform responsible for active Ca^{2+} extrusion (Dean 2010). In platelets the PMCA is subject to several regulatory influences (Dean 2010). The pump is stimulated by Ca^{2+}/calmodulin (Zabe and Dean 2001) and by protein kinase A-dependent phosphorylation (Dean et al. 1997), whilst tyrosine phosphorylation inhibits PMCA activity (Dean et al. 1997), an action that potentiates agonist-evoked rises in $[Ca^{2+}]_{cyt}$ and so platelet functional responses (Bozulic et al. 2007). The PMCA is also modulated by calpain-dependent proteolytic cleavage, with work in vitro and in erythrocytes showing removal of a C-terminal autoinhibitory domain increases activity, whereas further cleavage results in inactivation (Dean 2010). Similar cleavage patterns in human platelets suggest modulation of platelet PMCA by calpain (Brown and Dean 2007).

There are two types of plasma membrane Na^+/Ca^{2+} exchangers: those that exchange Na^+ for Ca^{2+} (NCXs) and those that exchange Na^+ for Ca^{2+} and K^+ (NCKXs) (Lytton 2007). Early functional studies suggested that human platelets possessed an NCKX (Kimura et al. 1993). Messenger RNA for three isoforms of NCKX are reported to be detectible in human platelets (Bugert et al. 2003), however only one study has reported detection of low levels of NCKX1 by Western blotting (Roberts et al. 2012), and others have concluded that NCKX isoforms are absent from the platelet proteome (Boyanova et al. 2012). Low levels of the cardiac NCX isoform, NCX1, have been reported in some Western blot studies (Roberts et al. 2012), whilst others have failed to detect this isoform in human platelets (Harper et al. 2010). A proteomic screen of platelet plasma membrane proteins (Lewandrowski et al. 2009) as well as Western blotting studies (Harper et al. 2010; Roberts et al. 2012) all suggest that NCX3 is the predominant NCX isoform in human platelets. NCX3, like the better studied NCX1, is believed to exchange three Na^+ ions for one Ca^{2+} ion (Lytton 2007). Some early studies concluded that NCXs played little role in influencing $[Ca^{2+}]_{cyt}$ in resting or stimulated platelets (Sage and Rink 1987; Rosado and Sage 2000), however more recent work has provided evidence that forward mode Na^+/Ca^{2+} exchange exports Ca^{2+} across the plasma membrane in resting and thrombin-stimulated human platelets (Roberts et al. 2012). The roles of NCXs in shaping platelet Ca^{2+} signals are complex, with

evidence indicating that these exchangers can promote a rise in $[Ca^{2+}]_{cyt}$ in stimulated platelets. It has been suggested that in collagen-stimulated platelets NCXs transiently reverse to mediate Ca^{2+} entry (Roberts et al. 2012), whilst in thrombin-stimulated platelets there is evidence that NCXs operating in forward mode promote Ca^{2+} recycling via the open canalicular system, supporting the Ca^{2+} signal directly and by promoting the secretion of autocoids from dense granules (Sage et al. 2013) (see section "Signal Magnitude").

Ca^{2+} Sequestration by Intracellular Organelles

Ca^{2+} is sequestered into the main releasable platelet Ca^{2+} store in the DTS by SERCA2b (Enouf et al. 1992), whilst Ca^{2+} is sequestered into the acidic Ca^{2+} stores that are probably located in the dense granules and lysosomes by SERCA3 (Wuytack et al. 1994). SERCA3 shows a much lower sensitivity to thapsigargin than SERCA2b, but is inhibited by 2,5-di-(*t*-butyl)-1,4-hydroquinone (TBHQ) (Cavallini et al. 1995). The acidic Ca^{2+} store membranes also contain a vacuolar H^+-ATPase (vH^+-ATPase), which may be coupled to a H^+/Ca^{2+} exchanger to facilitate Ca^{2+} loading (Sage et al. 2011). Evidence for such a SERCA-independent mechanism of Ca^{2+} sequestration comes from experiments in which the intracellular store $[Ca^{2+}]$ ($[Ca^{2+}]_{st}$) was monitored using Fluo-5 N and $[Ca^{2+}]_{cyt}$ was monitored using Fura-2 (Sage et al. 2011). SERCA2b and SERCA3 were inhibited using high concentrations of thapsigargin (1 μM) and TBHQ (20 μM) and non-acidic Ca^{2+} stores were depleted using the Ca^{2+} ionophore, ionomycin. Since ionomycin is a $Ca^{2+}/H+$ ionophore (Fasolato and Pozzan 1989; Fasolato et al. 1991), it is unable to deplete acidic Ca^{2+} stores due to the absence of a favourable electrochemical gradient for exchange. With SERCAs inhibited, ionomycin caused a rise in $[Ca^{2+}]_{cyt}$ and a fall in $[Ca^{2+}]_{st}$, changes which then gradually reversed indicating Ca^{2+} sequestration in the absence of SERCA activity (Sage et al. 2011). Addition of the pronophore, nigericin, after SERCA inhibitors and ionomycin, caused a further decrease in $[Ca^{2+}]_{st}$ and rise in $[Ca^{2+}]_{cyt}$ which did not reverse over several minutes, indicating that SERCA-independent sequestration was into acidic stores. The nature of the novel SERCA-independent Ca^{2+} sequestration mechanism remains to be identified, but a Ca^{2+}/H^+ exchanger coupled to vH^+-ATPase activity has been suggested on the basis of similarities between platelet dense granules and the acidosomes found in trypanosomes (Ruiz et al. 2004), where such a system operates (Vercesi et al. 1994). Another possibility is that a secretory pathway Ca^{2+}-ATPase may be involved, as found in the dense core vesicles of neuroendocrine cells (Mitchell et al. 2001). A further possibility is that the mitochondrial Ca^{2+} uniporter plays a role (Kirichok et al. 2004). However, the use of antimycin A and oligomycin to inhibit mitochondrial Ca^{2+} uptake does not affect changes in $[Ca^{2+}]_{cyt}$ in platelets treated with thapsigargin and ionomycin (Rosado and Sage 2000).

The SERCA-dependent and pH-dependent Ca^{2+} sequestration mechanisms found in human platelets appear to have different effects in shaping agonist-evoked Ca^{2+} signals (Sage et al. 2011). SERCA inhibition increases the peak of the initial agonist-evoked rise in $[Ca^{2+}]_{cyt}$ and fall in $[Ca^{2+}]_{st}$, whilst blocking pH-dependent Ca^{2+} sequestration using nigericin is without effect on early agonist-evoked changes in $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{st}$, but enhances the later plateau in $[Ca^{2+}]_{cyt}$. Hence pH-dependent Ca^{2+} sequestration into acidic stores appears to commence with some delay after the initial stimulus.

Ca^{2+} Buffering in the Platelet Cytosol

In addition to the Ca^{2+} transport mechanisms detailed earlier, the free $[Ca^{2+}]_{cyt}$ is also influenced by the binding of Ca^{2+} to cytosolic proteins. The intrinsic ability of cellular proteins to buffer agonist-evoked Ca^{2+} signals is often overlooked by many investigators despite the ability of these events to shape the spatiotemporal dynamics of Ca^{2+} signals (Schwaller 2010; Sabatini et al. 2001). However, Ca^{2+} buffering is likely to be essential to the platelet's ability to utilise Ca^{2+} as a second messenger, as influx of Ca^{2+} through a single channel has the potential to deliver a toxic dose of Ca^{2+} to the cell within a split second in the absence of any other systems to restrain Ca^{2+} accumulation (Sage et al. 2013). Estimates of the Ca^{2+}-binding capacity of the platelet cytosol indicate that it is particularly high, with increases in free Ca^{2+} accounting for only around 0.01–0.1 % of all the Ca^{2+} ions entering this cellular compartment (Sage et al. 2013; Valant et al. 1992). These results suggest that Ca^{2+} buffering is likely to play a significant role in modulating platelet Ca^{2+} signal dynamics and the downstream functional responses. Despite this, there has been no study into which molecular targets may underlie this heavy cytosolic Ca^{2+} buffering, although a proteomic study of human platelets has identified a number of candidate Ca^{2+}-binding proteins including calmodulin, S100 proteins, annexins and tubulin (Burkhart et al. 2012) that may be responsible.

Whilst our understanding of the molecular machinery that creates agonist-evoked Ca^{2+} signals in platelets is indebted to the use of fluorescent Ca^{2+} indicators, it is also important to appreciate that the introduction of these compounds artificially increases the Ca^{2+} buffering capacity of the cells. This in turn can markedly alter the spatiotemporal characteristics of any agonist-evoked Ca^{2+} signal (Sabatini et al. 2001). A recent study demonstrated that loading platelets with the commonly used, high-affinity cytosolic Ca^{2+} indicator, Fura-2, could essentially double the Ca^{2+}-binding capacity of the platelet cytosol when compared to

the lower affinity Ca^{2+} indicators, Fura-4F and Fura-FF (Sage et al. 2013). This effect on the buffering capacity of the platelet cytosol could be observed to directly buffer Ca^{2+} signals as well as indirectly interfering with Ca^{2+}-release from intracellular stores (Sage et al. 2013), demonstrating the potential for Ca^{2+} indicators to disrupt normal Ca^{2+} signalling processes. In addition, the introduction of fluorescent Ca^{2+} indicators has also been demonstrated to modulate platelet functional responses to aggregating stimuli (Hatayama et al. 1985; Lanza et al. 1987). Thus, whilst Ca^{2+} indicators may allow us to understand the basic molecular machinery that is responsible for the creation of Ca^{2+} signals, the enhanced Ca^{2+} buffering elicited by their introduction may interfere with the platelet's ability to modulate or respond to this Ca^{2+} signal by interfering with the signal's interaction with its downstream effector molecules. Thus, caution must be taken into account when interpreting the functional responses of platelets loaded with these indicators.

Modulation of Platelet Ca^{2+} Signals

Ca^{2+} signalling systems in cells are strongly non-linear, because in addition to their ability to be activated by agonists, the activity of individual Ca^{2+}-transporting proteins can also be modulated in a time-dependent manner by Ca^{2+}-dependent signalling pathways. In platelets the most studied example of Ca^{2+}-dependent modulation of Ca^{2+} signalling comes from the known role of protein kinase C (PKC) to reduce agonist-evoked rises in $[Ca^{2+}]_{cyt}$ (Lever et al. 2015). However, despite PKC being a well-known modulator of platelet Ca^{2+} signalling, the mechanisms by which it does so have been uncertain, with suggested potential mechanisms including actions on IP_3 production (Rittenhouse and Sasson 1985; Zavoico et al. 1985; King and Rittenhouse 1989; Connolly et al. 1986) to effects on PMCAs and SERCAs (Cavallini and Alexandre 1994; Pollock et al. 1987; Tao et al. 1992). This difficulty in identifying the mechanisms of such modulations relates to the difficulty in studying the effect of a signalling system which has many potential targets when measuring $[Ca^{2+}]_{cyt}$ alone. More recent analysis of the effects of PKC on agonist-evoked Ca^{2+} fluxes into and out of the platelet cytosol has demonstrated a role for Ca^{2+}-dependent conventional PKC isoforms in accelerating Ca^{2+} removal from the cytosol both via an effect on SERCA activity, as well as by indirectly affecting NCX-mediated Ca^{2+} removal via increased Na^+/K^+-ATPase activity (Lever et al. 2015). This ability of PKC to limit agonist-evoked Ca^{2+} signals has been previously shown to play a key role in reducing the development of a procoagulant phenotype in collagen- and thrombin-stimulated platelets (Strehl et al. 2007), thus demonstrating that Ca^{2+}-dependent feedback pathways may play a role in tuning platelet functional responses to physiological stimuli.

In addition to the ability of Ca^{2+} to modulate the generation of its own signal, other physiologically relevant signalling pathways are also able to engage in cross talk with the Ca^{2+} signalling system. These include the ability of endothelial-derived inhibitors such as prostacyclin and nitric oxide to trigger rises in cAMP and cGMP in the platelet which are able to inhibit platelet Ca^{2+} signalling through effects on IP_3-mediated Ca^{2+} release, and possibly via effects on Ca^{2+} entry (for review see Smolenski 2012). In addition to other chemical signals, it has also been shown that shear stress can play a role in upregulating platelet Ca^{2+} signals, both in isolation of other chemical stimuli (Nesbitt et al. 2009) as well as in platelets translocating over surfaces coated with adhesive ligands (Goncalves et al. 2005; Mazzucato et al. 2002).

Controlling Stimulus–Response Coupling of Ca^{2+} Signals in Human Platelets

Calcium Effectors

The central role of Ca^{2+} in mediating platelet activation is through the ability of this second messenger system to translate extracellular signals into functional responses in these cells. The Ca^{2+} signalling system is therefore responsible for triggering and coordinating a range of different processes required for thrombus formation. To mediate this change in platelet activity, the cells require Ca^{2+}-sensitive effector proteins that can translate Ca^{2+} signals into appropriate intracellular responses. Platelets are known to possess a number of such effector proteins: Calmodulin, scinderin, gelsolin and calpain are involved in reorganisation of the actin cytoskeleton and platelet shape change (Paul et al. 1999; Witke et al. 1995; Rodríguez Del Castillo et al. 1992; Croce et al. 1999); CalDagGEFI and CIB1 are involved in integrin $\alpha_{IIb}\beta_3$ activation (Naik et al. 2009; Stefanini et al. 2009); PKC, Munc13-4, SNARE proteins and calpain are involved in granule secretion (Strehl et al. 2007; Golebiewska and Poole 2014; Croce et al. 1999); $cPLA_2$ underlies thromboxane production (Murthy et al. 1995) and TMEM16F is responsible for phosphatidylserine exposure (Mattheij et al. 2015).

The presence of a variety of Ca^{2+}-sensitive signal transduction pathways in one cell requires mechanisms to ensure coordinated activation of these pathways at the correct time and place to produce the desired functional response to any particular extracellular signals. If all the different Ca^{2+}-sensitive processes were fully activated in every platelet then a self-propagating cycle of platelet activation, incorporation into a forming thrombus, secretion of autocrine stimulants and recruitment of additional platelets would lead to uncontrolled thrombus growth, which could eventually occlude the vessel leading to deleterious consequences for the tissue downstream. However, thrombus formation in vivo can be

seen to be a self-limiting process, which does not lead to occlusion of the damaged vessel (Furie and Furie 2008). This suggests that there are a range of regulatory mechanisms that tightly control the ability of Ca^{2+} signals to activate their effector systems. Understanding the systems that prevent excessive activity may provide a valuable asset in devising treatments to prevent excessive blood clotting from occurring (Brass et al. 2011). Recent work has demonstrated that there is heterogeneity in platelet responses during thrombus formation (Munnix et al. 2007; Stalker et al. 2013; London et al. 2006), thus indicating that Ca^{2+}-stimulated processes are able to be differentially activated within the developing platelet aggregate. Whilst this can be explained through spatial control of the physical and chemical stimuli which individual platelets are exposed to when encountering the thrombus (Stalker et al. 2013; Nesbitt et al. 2009; Smolenski 2012), there is still a requirement for the platelet to translate these extracellular signals into a Ca^{2+} signal which can itself be translated into the selective activation of the various Ca^{2+} regulated processes found in these cells. How can individual platelets translate the Ca^{2+} signals they generate to elicit distinguishable phenotypes?

The versatility of Ca^{2+} signalling in cells comes from the ability of cells to selectively decode different agonist-evoked rises in $[Ca^{2+}]_{cyt}$ into specific cellular responses leading to responses over a remarkable range of different timescales (Berridge et al. 2003). The simplest Ca^{2+} signal available in this cellular language is a single Ca^{2+} transient, which can be seen to possess three distinct parameters that can be decrypted by the cell: the signal magnitude, its duration and its subcellular localisation. Previous work in other cells has demonstrated that Ca^{2+}-sensitive effector proteins with different Ca^{2+}-binding affinities and kinetics are able to selectively decode each of these parameters (Dolmetsch et al. 1997).

Signal Magnitude

In platelets, early work identified a role for gradually increasing thresholds of $[Ca^{2+}]_{cyt}$ for the activation of platelet shape change (400–500 nM), serotonin secretion (800 nM) and platelet aggregation (2 μM) (Rink et al. 1982; Hallam et al. 1985). These data establish the potential for the differential Ca^{2+} affinity of effector proteins in mediating these responses in platelets, yet the use of Ca^{2+} ionophores to artificially create Ca^{2+} signals of different amplitude does lead to questions over the physiological relevance of these early studies. Both studies demonstrated that stimulation with physiological agonists such as thrombin and platelet-activating factor could trigger similar responses below the $[Ca^{2+}]_{cyt}$ thresholds defined with ionophores, suggesting that additional regulatory mechanisms were also triggered by agonist stimulation. In addition to other known signalling pathways that may sensitise responses to elevated $[Ca^{2+}]_{cyt}$ (e.g. Paul et al. 1999), it may be that Ca^{2+} microdomains elicited in the vicinity of Ca^{2+}-permeable ion channels play a role in facilitating stimulation of Ca^{2+}-dependent processes in platelets even when the average $[Ca^{2+}]_{cyt}$ reported by fluorescent indicators is relatively low (Parekh 2008).

Signal Duration

The duration of the agonist-evoked rises in $[Ca^{2+}]_{cyt}$ may play an important role in determining important shifts in the functional properties of platelets. Previous work examining platelet interaction with VWF under flow conditions has shown that the duration of Ca^{2+} signalling is crucial in determining the final physiological response of these cells (Nesbitt et al. 2002; Mazzucato et al. 2002). Both of these studies demonstrated that the duration of platelet arrest onto a VWF-coated surface was determined by the length of the Ca^{2+} transient. These transients were not dependent on the presence of extracellular Ca^{2+} and were therefore dependent upon Ca^{2+} release from intracellular stores. In contrast, when the Ca^{2+} signals were prolonged oscillatory responses dependent on Ca^{2+} entry, they led to integrin-dependent, irreversible adhesion and platelet aggregation. This relationship between Ca^{2+} signalling and incorporation within a platelet aggregate was also demonstrated in vivo by van Gestel et al. (2002). This group demonstrated that platelets that were observed to have a sustained increase in their $[Ca^{2+}]_{cyt}$ after binding to the surface of a growing thrombus become stably incorporated within this structure, whilst platelets whose $[Ca^{2+}]_{cyt}$ returned to baseline were found to embolise. Beyond a role in primary haemostatic reactions, prolongation of platelet Ca^{2+} signals has also been demonstrated to play a key role in regulating the blood coagulation process (Heemskerk et al. 2013). Activated platelets create a catalytic surface for the activation of the tenase and prothrombinase complexes through the expression of anionic phospholipids such as phosphatidylserine on the extracellular face of their plasma membrane. Previous work has demonstrated that a large, sustained increase in $[Ca^{2+}]_{cyt}$ is key to the ability of platelets to develop a procoagulant phenotype (Heemskerk et al. 1997; Kulkarni and Jackson 2004; Smeets et al. 1993; Jackson and Schoenwaelder, 2010).

As can be seen from the earlier discussion, prolonged Ca^{2+} signalling in platelets plays a role in determining the rate and extent of both the primary and secondary haemostatic systems that they elicit. Therefore, blocking aspects of the Ca^{2+} signalling system that could elicit a shortening of cytosolic Ca^{2+} signals may be useful for limiting the extent of unwanted platelet aggregation in thrombotic conditions.

A number of signalling pathways are important in eliciting a large, sustained increase in $[Ca^{2+}]_{cyt}$ in agonist-stimulated platelets. These include key roles for store-operated- and receptor-operated Ca^{2+} entry pathways

(Gilio et al. 2010; Harper et al. 2013), autocrine stimulation by the contents of the dense granules (Weiss and Lages 1997; Lages and Weiss 1999) and the activity of Na^+/Ca^{2+} exchangers (Harper et al. 2013; Sage et al. 2013). However, how these systems are coordinated to control the maintenance of agonist-evoked Ca^{2+} signals is less clear. Recent studies have suggested a unified mechanism for prolonging Ca^{2+} signalling via a pericellular Ca^{2+} recycling system. This work has suggested that Ca^{2+} removed from the cell by the NCX can accumulate to a high concentration within the narrow confines of the open canalicular system, such that it can then be recycled back into the cell down its concentration gradient through Ca^{2+}-permeable ion channels and possibly by reverse mode NCX activity, thus prolonging Ca^{2+} signalling directly as well as indirectly through triggering dense granule secretion (Sage et al. 2013; Walford et al. 2015). Further work will be required to define whether this system could be responsible for determining if a platelet adopts proaggregatory and procoagulant phenotypes during thrombus formation under physiological flow conditions.

Subcellular Localisation

In other cells selective activation of Ca^{2+}-sensitive signalling pathways can be achieved through tight spatial coupling of Ca^{2+}-permeable channels and Ca^{2+}-sensitive effectors in specific microdomains of the cell (Rizzuto and Pozzan 2006). Yet the size of the resting platelet is comparative in size or smaller than the spatial spread of elementary Ca^{2+} signals generated through the isolated opening of individual groups of ion channels in other cells (Niggli and Shirokova 2007; Sage et al. 2013). This would seem to suggest that Ca^{2+} signals should always spread throughout the entire platelets. However, Ca^{2+} microdomains may be masked in the tiny platelet volume by the ability of high-affinity, highly diffusible fluorescent Ca^{2+} indicators to act as highly mobile shuttles for rapid transport of Ca^{2+} through the cell (Sala and Hernandez-Cruz 1990). Similar distortions have previously been reported in the spines of Purkinje neurons (Schmidt et al. 2007). In spite of this, previous single cell imaging studies have reported the presence of either localised cytosolic Ca^{2+} microdomains or Ca^{2+} gradients across human platelets (Ariyoshi and Salzman 1996; Tsunoda et al. 1988). In addition, Nesbitt et al. (2009) reported that a localised Ca^{2+} signal was generated that allowed membrane tether formation in adherent platelets, without notable activation of shape change or aggregatory mechanisms. These data suggest that platelets can significantly restrict Ca^{2+} diffusion from the source of entry into the cytosol, and thus use the subcellular localisation of Ca^{2+} signals to selectively activate some Ca^{2+} effectors.

To create localised rises of Ca^{2+} in specific areas would require platelets to localise Ca^{2+}-transporting processes to specific subregions of the cell. This possibility is suggested by a number of previous studies which have shown a key role for the platelet cytoskeleton (Ariyoshi and Salzman 1996, Walford et al. 2015) and lipid raft domains (Brownlow et al. 2004) in facilitating normal agonist-evoked Ca^{2+} signals. Single cell imaging studies have also found evidence for specific subregions of platelets being the sites of Ca^{2+} mobilisation (Heemskerk et al. 2002) as well as Ca^{2+} removal from the platelet cytosol (Sage et al. 2013), suggesting that Ca^{2+}-transporting proteins are inhomogenously located in these cells. In addition to locating Ca^{2+} channels and exchangers in specific regions, platelets would also require cellular mechanisms to restrict the spatial spread of Ca^{2+} through the cytosol to account for the Ca^{2+} microdomains and gradients described earlier. Recent work has suggested that the membrane complex, formed by close association of the open canalicular system with the dense tubular system, may create a suitable cellular architecture to generate such localised Ca^{2+} signals in platelets (Walford et al. 2015). The close association of both the open canalicular system and the dense tubular system at the membrane complex creates a nanojunction, which in other cell types has been shown to play a role in creating a region of the cytosol in which Ca^{2+} concentration can be controlled in isolation from the rest of the cell (van Breemen et al. 2013; White 1972). The possibility that the membrane complex may control platelet Ca^{2+} signalling has been suggested by the identification of a family with a bleeding disorder characterised by the lack of a membrane complex and a defect in thrombin-evoked Ca^{2+} signalling (Parker et al. 1993).

Coincidence Detection

Although individual platelet function may be affected principally by the Ca^{2+} signal within its cytosol, it has also become apparent that the outcome of that signal is also dependent on whether other platelets encountered on the surface of the thrombus are also simultaneously active. Previous work has demonstrated that thrombus growth is dependent on the presence of a coincident Ca^{2+} signal in a neighbouring cell (Nesbitt et al. 2003). Although intracellular Ca^{2+} signalling may facilitate thrombus growth, recent work has also suggested that Ca^{2+} signalling can also upregulate nitric oxide production in platelets, which may work to prevent thrombus growth (Cozzi et al. 2015). Thus, further research will be necessary to understand how these distinct pathways interact to regulate the Ca^{2+}-dependent growth of a thrombus.

Whilst we now have a reasonable understanding of the molecular machinery involved in mediating agonist-evoked rises in $[Ca^{2+}]_{cyt}$, there is still much to learn about how the channels and transporters concerned work to alter the

spatiotemporal patterns of Ca^{2+} signals, how these Ca^{2+} signals regulate downstream Ca^{2+}-sensitive processes and how these systems work alongside other signalling pathways to help regulate both the growth of a thrombus and the patterning of individual platelet responses within it.

Take Home Messages

- A rise in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) is central to platelet activation.
- Agonists stimulate a rise in $[Ca^{2+}]_{cyt}$ by releasing Ca^{2+} from intracellular stores and stimulating Ca^{2+} entry across the plasma membrane.
- Stored Ca^{2+} may be released from the dense tubular system (DTS) and acidic organelles by the second messengers inositol 1,4,5-trisphosphate (IP_3) and nicotinic acid adenine dinucleotide phosphate (NAADP), respectively.
- Ca^{2+} entry across the plasma membrane may be activated in response to store depletion (store-operated Ca^{2+} entry; SOCE), in response to the second messenger diacylglycerol (DAG) or by activation of an ionotropic receptor (ATP acting at the P2X1 receptor).
- Platelet activation may increase $[Ca^{2+}]_{cyt}$ from 50 to 100 nM to several μM and result in oscillations or repetitive spikes in elevated $[Ca^{2+}]_{cyt}$ in individual cells.
- Imaging studies have revealed platelet Ca^{2+} signals show subcellular microdomains and the platelet membrane complex may form a specialised nanojunction for Ca^{2+} released by the DTS to be removed into the open canalicular system for subsequent recycling back to the cytosol.
- Platelets Ca^{2+} signals are transduced into function responses by many effector proteins including calmodulin, scinderin, gelsolin, calpain, CalDagGEFI, CIB1, PKC, Munc13-4, SNARE proteins, $cPLA_2$ and TMEM16F.
- Ca^{2+} is removed from the platelet cytosol by sequestration into organelles and removal across the plasma membrane.
- Ca^{2+} is sequestered by sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) and by a vacuolar H^+-ATPase (vH^+-ATPase), probably coupled to a H^+/Ca^{2+} exchanger.
- Ca^{2+} is removed across the plasma membrane either by primary active transport via plasma membrane Ca^{2+}-ATPases (PMCAs), and by Na^+/Ca^{2+} exchangers.

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Platelet Signaling: Protein Phosphorylation

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Abstract

Protein phosphorylation is the main mechanism by which platelet functional responses are regulated. There are myriad protein kinases in the platelet, which are classified by the residue in which they phosphorylate. Protein tyrosine kinases phosphorylate tyrosine residues, while protein serine/threonine kinases phosphorylate serine or threonine residues. Several important signaling cascades are initiated upon platelet activation. Here we will explore the relevance of phosphorylation of specific proteins important to several essential signaling cascades in platelets.

Protein phosphorylation is the major molecular mechanism through which protein function is regulated in response to extracellular stimuli in platelets. Virtually all types of extracellular signals that activate platelets produce most of their diverse physiological effects by regulating phosphorylation of specific phosphoproteins. Although proteins may be covalently modified in many other ways, including acylation (acetylation, myristoylation, isoprenylation), carboxymethylation, tyrosine sulfation, and glycosylation, none of these mechanisms are nearly as widespread as protein phosphorylation. Among the various types of posttranslational modifications, protein phosphorylation is the most important one, regulating the structures and functions of cellular proteins in a wide spectrum of platelet processes, including aggregation, secretion, spreading, and clot retraction. Thus, protein phosphorylation is by far the most prominent mechanism through which platelets regulate their functional responses.

Today, it is believed that one-third of all proteins in the cell are phosphorylated at one time or another, and half of these proteins likely harbor more than 1 phosphorylation site, with phosphorylation at different sites often eliciting quite different cellular responses. Protein phosphorylation events in platelets are regulated by protein kinases. Protein kinases phosphorylate proteins by transferring the γ-phosphate of ATP to tyrosine, serine, or threonine residues. These protein kinases are divided into two groups, tyrosine kinases and serine/threonine kinases, depending on which residue the kinase phosphorylates. Both groups of enzymes are phosphotransferases; they catalyze different reactions to modulate the structures and functions of many cellular proteins in platelets.

Two major mechanisms are involved in receptor-mediated platelet protein phosphorylation. In one of the mechanisms the extracellular signals, or first messengers, regulate protein kinases such as Src Family kinases (SFKs), Syk, or Btk indirectly by acting on plasma membrane receptors such as immunoglobulin-like receptors and GPCRs, thereby regulating the intracellular concentration of a second messenger in platelets. Prominent second messengers, such as Ca^{2+} and diacylglycerol (DAG) in the platelet system, activate protein kinases. The next steps in these pathways are the activation of specific classes of protein serine/threonine kinases (Protein Kinase C) by these second messengers and the subsequent phosphorylation of specific substrate proteins, which leads to platelet activation. In this chapter, we present an overview of the vital role of protein phosphorylation events and their regulation by different protein kinases on platelet function.

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Receptor Phosphorylation Regulates Its Function in Platelets

Platelet receptor phosphorylation plays an important role not only in the activation of the receptor but also its regulation. Platelets mainly express two different types of agonist receptors, G-protein-coupled receptors (GPCRs) and tyrosine kinase pathway receptors, both of which are important for platelet activation (Offermanns 2000; Sambrano et al. 2001). There are two different tyrosine kinase-coupled receptors. One is the ITAM (Immunoreceptor tyrosine-based activation motif)-containing receptors, such as the Fc receptor-γ chain (FcRγ) and FcγRIIA, which has the classic YXX (L/I) X_{6-12} YXX (L/I) with two tyrosine residues in its cytoplasmic domain. The other is a hemITAM-containing receptor, such as CLEC-2, which has only one tyrosine residue (YXX(L/I)) in its cytoplasmic domain (Fuller et al. 2007; Underhill and Goodridge 2007). These tyrosine residues in the cytoplasmic domain of FcRγ, FcγRIIA, and CLEC-2 are phosphorylated by Src family kinases when platelets are stimulated with the respective agonists. Using knockout mouse models as well as cell lines which express receptors mutated at these tyrosine residues demonstrates that phosphorylation of these tyrosine residues is important for ITAM and hemITAM receptor activation and downstream signaling (Fuller et al. 2007). However, unlike tyrosine kinase receptors, phosphorylation of GPCRs on serine and threonine residues causes receptor desensitization thereby regulating their activity. Two types of kinases are known to phosphorylate and desensitize the GPCRs in other cells. One type—the second-messenger-dependent kinases, such as cAMP-dependent protein kinase and protein kinase C and protein kinase G—alters the receptor conformation such that the affinity of the receptor for the G protein is greatly reduced and also helps in heterologous receptor desensitization (Kelley-Hickie et al. 2007; Homma and Hanahan 1988). Heterologous receptor desensitization occurs after prolonged activation by one agonist and results in the inability of another agonist to cause activation. The second type of kinase—GPCR kinases (GRKs)—is involved in homologous receptor desensitization. This desensitization is referred to as homologous because only agonist-occupied receptors are substrates for GRKs, ensuring that only those receptors that have been activated will be desensitized. Relatively little is known about the role of GRKs in platelets and megakaryocytes (Kelley-Hickie and Kinsella 2006).

Src Family Kinases

Src family kinases (SFKs) play a central role in mediating the rapid response of platelets to vascular injury (Newman 2009; Senis et al. 2014). This rapid response of platelets to vascular injury is mediated by many tyrosine kinase-linked receptors. Ligand-mediated clustering of these receptors triggers transmission of primary activation signals in platelets. However, none of these receptors have intrinsic kinase activity, so they rely on a family of protein tyrosine kinases called Src family kinases. These kinases are either associated or in close proximity to the tyrosine residues on the receptor cytoplasmic tails in order to phosphorylate the tyrosine residues that mediate platelet activation (Ezumi et al. 1998; Suzuki-Inoue et al. 2002). Activated SFKs are also known to phosphorylate enzymes, adaptors, and cytoskeletal proteins that collectively propagate the signal and coordinate platelet activation, which enhances thrombus growth and stability. SFKs such as Src, Lyn, and Fyn are highly expressed and well studied in platelets and regulate platelet activation by GPVI, CLEC-2, FcγRIIA, GPIb-IX-V, and $\alpha_{IIb}\beta_3$ (Briddon and Watson 1999; Quek et al. 2000; Arias-Salgado et al. 2003; Yin et al. 2008; Chari et al. 2009b). Src and Fyn are primarily regarded as positive regulators, whereas Lyn plays a dual role as a positive and a negative regulator of platelet activation (Fig. 1). All the three SFKs also contribute to G_q- and G_i-coupled receptor signaling (Fig. 2) (Garcia et al. 2005, 2007; Kim et al. 2006; Canobbio et al. 2015). A number of structurally distinct broad-spectrum SFK inhibitors (PP1, PP2, PD0173952) and knockout mouse models have been used to elucidate the contributions of these SFKs to platelet signal transduction and function (Briddon and Watson 1999; Quek et al. 2000; Arias-Salgado et al. 2003; Yin et al. 2008; Chari et al. 2009b). The most well-established functions are summarized in Table 1.

Syk

Syk (spleen tyrosine kinase), a 72-kDa tyrosine kinase was first identified in thymus and spleen (Fluck et al. 1995). The Syk activation process is initiated when SFKs phosphorylate conserved tyrosine residues within ITAMs. These phosphotyrosine motifs are bound by Syk kinases via interaction with the tandem SH2 domains of the kinase. Binding of Syk kinases to ITAM sequences serves both to activate kinase activity and localize the kinase near its substrates (Gibbins et al. 1996). Once bound to the motifs Syk gets phosphorylated at multiple tyrosine residues and becomes active. The individual roles of these tyrosine residues are still not clearly understood. In all cases, it appears that receptor stimulation results in recruitment of Syk to ITAM sequences although the sequence and spacing of ITAMs vary between different receptors. For example, in hemITAM receptor activation, Syk activation is dependent on both SFKs and Tec family kinases (Manne et al. 2015). It was also observed that tyrosine phosphorylation on Syk is differentially regulated in human and murine platelets by protein kinase C isoforms, especially by PKC-β in human platelets

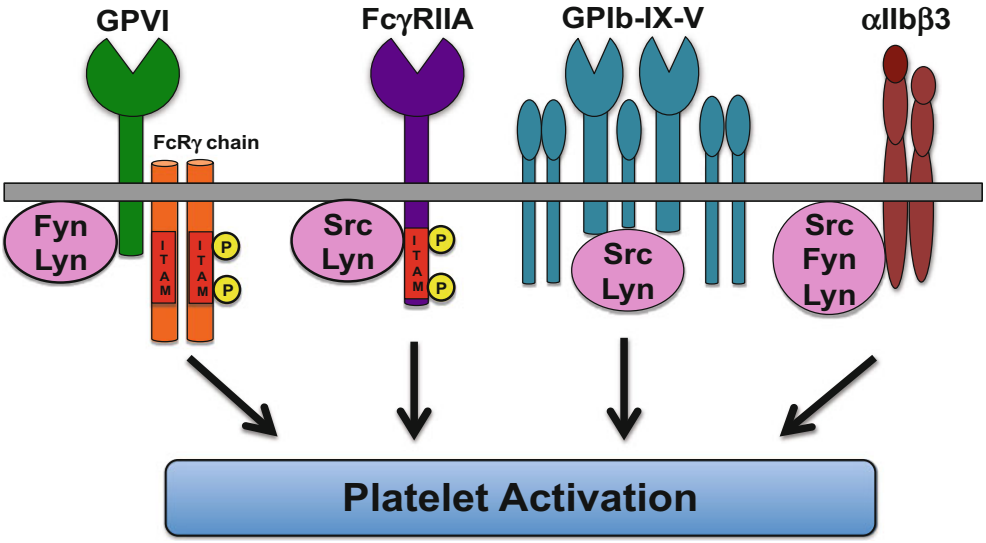

Fig. 1 Role of Src family kinases in platelet activation

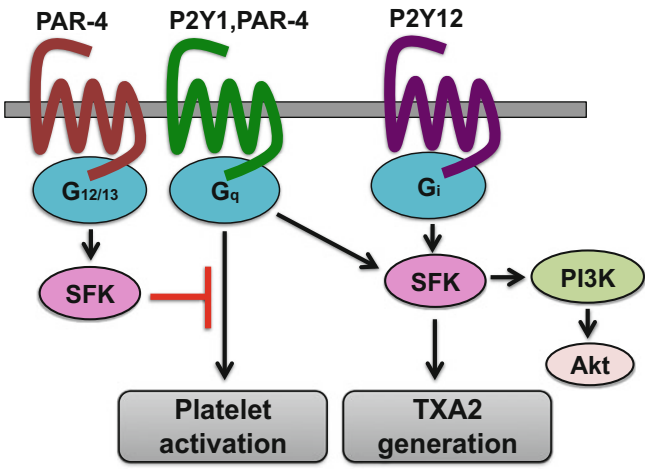

Fig. 2 Role of Src family kinases downstream of G protein-coupled receptors in platelets

Table 1 Functional role of Src family kinases in platelets

Src family kinases	Receptor signaling regulated by SFKs
Src	Positively regulates $\alpha_{IIb}\beta_3$-mediated signaling and functional responses
Fyn	Positively regulates GPVI-mediated signaling and aggregation/secretion
	Potentiation of PAR-4-mediated aggregation
Lyn	Increases $\alpha_{IIb}\beta_3$-mediated spreading
	Delayed onset of GPVI-mediated signaling and aggregation/secretion
	Positively regulates GPIb-IX-V-mediated spreading

(Buitrago et al. 2013). LAT and SLP76 are the major direct downstream substrates of Syk as phosphorylation of both of these substrates is abolished in Syk−/−platelets (Suzuki-Inoue et al. 2006). Recent studies using Syk knockin model confirm a critical role of the N-terminal SH2 domain of a Syk in ITAM and hemITAM signaling (Hughes et al. 2015). This study also shows that the phosphorylation of Syk on Y348 and of the CLEC-2 hemITAM is upstream of auto-phosphorylation of two tyrosine residues, 519/520. $Syk^{-/-}$ platelets were deficient in functional responses with GPVI, CLEC-2, and FcγRIIA (Hughes et al. 2015; Suzuki-Inoue et al. 2006). Interestingly, Syk knockout mice die prenatally due to severe hemorrhaging, also suggesting a role for Syk in maintaining vascular integrity. Recent studies have shown that Syk activation downstream of CLEC-2 plays a crucial role in blood-lymphatic vessel separation and maintenance (Hess et al. 2014; Bertozzi et al. 2010). A number of structurally distinct Syk inhibitors (OXSI2, PRT318, R406) (Bhavaraju et al. 2008; Spalton et al. 2009; Reilly et al. 2011) and knockout mouse models have been used to elucidate the contribution of Syk to platelet signal transduction and function.

Tec Family Kinases

Tec family kinases are known to regulate PLCγ2 in B and T cells (Lewis et al. 2001). The structure of these enzymes shows that, in addition to containing SH2 and SH3 domains, they have a unique pleckstrin (PH) domain, which plays an important role in activation of these kinases by SFKs (Okoh and Vihinen 1999; Mohamed et al. 2009). The Tec family of kinases consists of five different kinases: Bmx, Btk, Itk, Tec, Txk/Rlk (Schmidt et al. 2004). Of these kinases, Btk and Tec are highly expressed in platelets (Atkinson et al. 2003). Activation of Tec family tyrosine kinases occurs downstream of both Src and Syk family kinases in signaling pathways in platelets (Manne et al. 2015). Upon signal initiation, most Tec family kinases translocate to the plasma membrane

through binding of their pleckstrin homology (PH) domain to phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) created by the activation of PI 3′-kinase. At the membrane Tec family kinases become phosphorylated within their activation loop (Tyr 551 in Btk) via either Src family kinases or auto-phosphorylation. Another site that becomes phosphorylated is within the SH3 domain of the kinase (Tyr 223 in Btk). In platelets, Btk and Tec proteins are phosphorylated in response to GPVI and CLEC-2 agonists (Manne et al. 2015; Atkinson et al. 2003) (Fig. 3). The role of Btk in platelets was studied extensively by using platelets from knockout mice and X-linked agammaglobulinemia (XLA) patients, a disease condition in which Btk is dysfunctional (Futatani et al. 2001; Liu et al. 2006). PLCγ2 phosphorylation downstream of GPVI has been show to be regulated by both Btk and Tec kinases (Atkinson et al. 2003). Studies of platelets from mice deficient in Btk and Tec have shown that these proteins are crucial for CLEC-2 signaling, which plays a crucial role in blood-lymphatic vessel separation and maintenance (Manne et al. 2015). Ibrutinib, a Btk inhibitor, is used for treatment in human patients with chronic lymphocytic leukemia (CLL) (Akinleye et al. 2013). Recent phase 3 clinical studies using Ibrutinib showed that a small percent of patients have bruises on the skin, a characteristic of lack of platelet activation (Akinleye et al. 2013). It is also shown that CLL patients treated with Ibrutinib have defects in collagen and vWF-mediated platelet activation, which supports the important role of Btk in platelets (Kamel et al. 2015).

Serine/Threonine Kinases

Serine/threonine kinases catalyze phosphorylation of serine or threonine residues on their target proteins. Protein kinase C, Protein kinase G, Protein kinase A, Protein kinase D, Protein kinase B (Akt), casein kinase, and Erk are some of the ser/thr kinases expressed in platelets.

Protein Kinase C Protein kinase Cs are a subfamily belonging to AGC kinase superfamily. PKC isoforms were first discovered as being activated by diacylglycerol (DAG), a lipid product present in the cell membrane (Mori et al. 1982). PKC isoforms have an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain. PKC isoforms are divided into three subclasses based on their structures and cofactor requirements. A model depicting the structure of PKC isoforms is shown in Fig. 4. Classical PKC isoforms (α, βI, βII, γ) have both C1 and C2 domains and hence depend on calcium and diacyl glycerol (DAG) for their activation; the members of a novel class of PKC isoforms (δ, θ, ε, η) have a C2-like domain that cannot bind calcium and hence are calcium insensitive but require DAG for their activation. Atypical PKC isoforms (ι/λ, ζ) have a C1-like domain that cannot bind DAG and lack a C2 domain. Hence atypical PKC isoforms are both calcium and DAG insensitive but depend on phospholipids for their activation. Among these, PKC isoforms α, β, δ, η, θ, ε and ζ are present in platelets (Crosby and Poole 2003; Murugappan et al. 2004). Under resting conditions, PKC isoforms remain in a closed conformation with the pseudo-substrate-binding domain occupying the substrate-binding site in the C4 domain. Upon platelet agonist stimulation, calcium mobilization and DAG generation lead to translocation and activation of PKC isoforms (Steinberg 2004). Upon activation, PKC isoforms through receptors for activated C kinases (RACKs) localize to their respective substrates and cause substrate phosphorylation and downstream signaling events (Harper and Poole 2010). Dr Mochly-Rosen's group has shown that RACKs are PKC specific and were exploited to design isoform-specific PKC inhibitors (Yedovitzky et al. 1997).

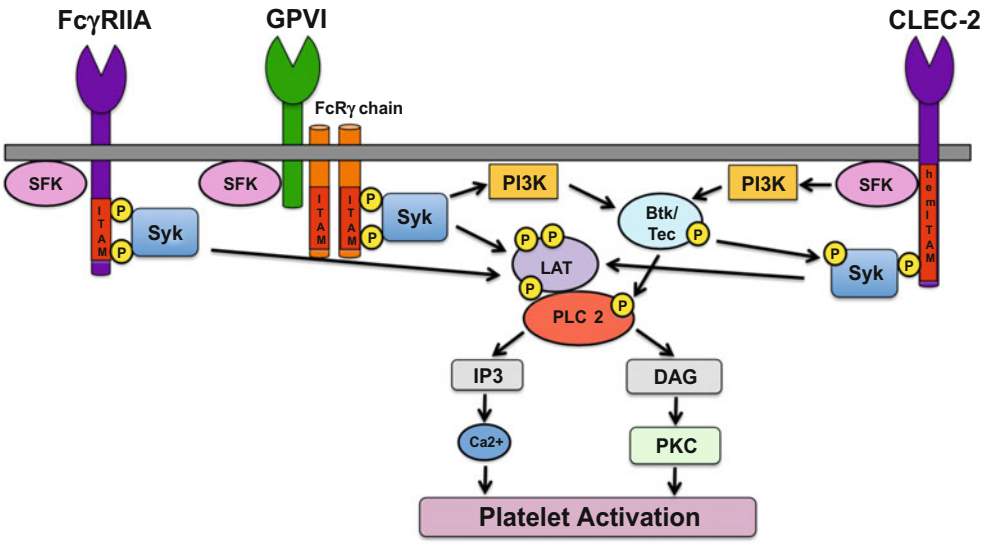

Fig. 3 Role of Syk and Tec family kinases in platelet activation

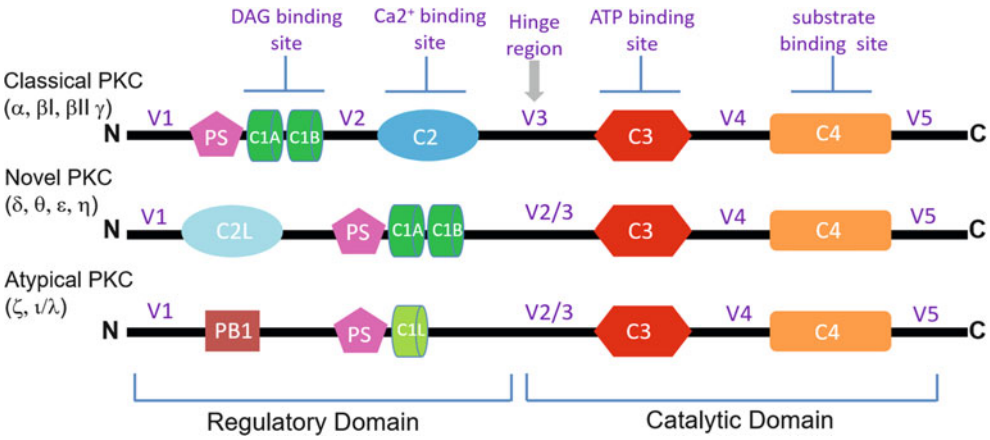

Fig. 4 Model depicting structure of PKC isoforms. *PS* pseudosubstrate domain, *C* constant domains, *V* variable domains; *C2L* C2 like domain, *C1L* C1 like domain, *PB1* Phox and Bem1 domain

PKC isoforms are generally considered active when the threonine residue in their hydrophobic motif gets phosphorylated. Since phosphorylation is the main mechanism through which PKC isoforms are activated, several ATP-competitive inhibitors such as Go6976 (Martiny-Baron et al. 1993), bisindolemaleimide I (Balaban et al. 1999), GF109203X (Toullec et al. 1991), and Ro812230 (Beltman et al. 1996), were used to study their role in vitro and isoform-specific knockout mice were used to study their role in vivo. AEB071, a PKC inhibitor has been shown to have preclinical activity in CLL (El-Gamal et al. 2014) and is currently under clinical trial for treating melanoma (clinicaltrials.gov).

Classical PKC Isoforms PKCα regulates α-granule biogenesis, secretion, dense granule release, and thrombus formation with no defect in hemostasis observed in animals deficient in the enzyme (as shown by tail bleeding times) (Konopatskaya et al. 2009).

PKCβ associates with integrin $\alpha_{IIb}\beta_3$ and regulates platelet spreading on immobilized fibrinogen (Buensuceso et al. 2005). PKCβ also regulates platelet spreading on collagen (Gilio et al. 2010) and is implicated in negatively regulating GPVI-mediated Syk tyrosine phosphorylation in human but not murine platelets (Buitrago et al. 2013). Enzastaurin, a PKCβ inhibitor was shown to have anticancer activity. However, the drug was also shown to potentiate platelet aggregation and growth factor secretion which may be detrimental to patients (Lesyk et al. 2015). It is currently under clinical trials for treating different cancers (clinicaltrials.gov). The role of classical PKC isoforms is depicted in Fig. 5.

Novel PKC Isoforms PKCδ, upon phosphorylation of thr505 in its activation loop, becomes active and is implicated in negatively regulating GPVI-mediated platelet functional responses by associating with SHIP1 and Lyn whereas it positively regulates PAR-mediated platelet responses including granule secretion and thromboxane generation (Chari et al. 2009a). However, PKCδ null mice do not have an in vivo thrombotic defect in $FeCl_3$-induced injury model possibly owing to the fact of opposing effects of PKCδ to different agonists. Besides, PKCδ is also phosphorylated on tyrosine residues downstream of PAR and GPVI receptors (Moussazadeh and Haimovich 1998; Crosby and Poole 2003). Human and mouse PKCδ protein contain 20 and 19 tyrosine residues, respectively. Phosphorylation on tyrosine residues 52, 64, 155, 187 (present in regulatory domain); residues 311 and 332 (present in the hinge region); and residues 512, 523, and 565 (present in catalytic domain) were reported so far (Steinberg 2004). Phosphorylation of PKCδ on Y565 potentiates the activity of the kinase (Hall et al. 2007). Y311 phosphorylation on PKCδ has been implicated in PAR-mediated thromboxane generation (Murugappan et al. 2005) and ADP-induced thromboxane generation (Bhavanasi et al. 2015). Y311-phosphorylated PKCδ has been shown to have lipid-independent kinase activity and this change in cofactor requirements is also accompanied by change in substrate specificity (Bhavanasi et al. 2004, 2015). Studies in RBL-2H3 cells have identified Lyn-dependent phosphorylation of PKCδ at Y332 and that this phosphorylation mediates association of PKCδ with Shc (Leitges et al. 2002) regulating mast cell degranulation indicating that PKCδ could act both as a kinase and as an adaptor. A peptide inhibitor of PKCδ, based on the RACK sequence, was developed and was shown to inhibit reperfusion injury in ischemic heart (Chen et al. 2001; Inagaki et al. 2003) as well as inhibiting platelet function (Chari et al. 2009a).

PKCθ becomes active upon phosphorylation of its thr538 residue in platelets. It is a positive regulator of PAR4-mediated platelet responses (Nagy et al. 2009). The role of PKCθ in GPVI-mediated platelet activation is complicated as it negatively regulates GPVI responses at low concentrations of the agonist but positively regulates GPVI responses at high concentrations of the agonist (Nagy et al. 2009; Harper and Poole 2009). PKCθ null mice have

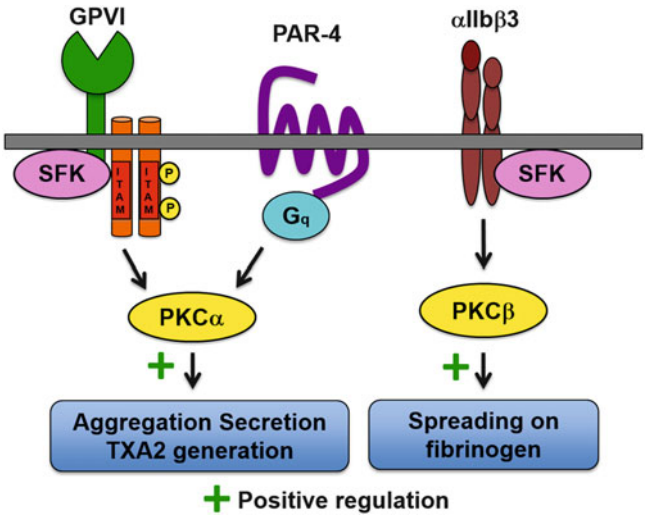

Fig. 5 Role of classical PKC isoforms in regulating platelet activation

delayed thrombus formation in $FeCl_3$-induced injury model and flow over collagen suggesting a role for PKCθ in regulating pathological thrombus formation (Hall et al. 2008; Nagy et al. 2009). PKCθ, upon phosphorylation on its Y90 residue, is also shown to be active in T cells (Liu et al. 2000) and the activity of PKCθ is required for T-cell activation and survival (Sun et al. 2000). PKCθ was also shown to regulate platelet outside-in signaling by being tyrosine phosphorylated but the exact tyrosine phosphorylation site is not known. It regulates platelet spreading on fibrinogen by phosphorylating WASP (Wiskott–Aldrich syndrome protein) interacting protein on ser488 that regulates actin polymerization (Soriani et al. 2006).

Agonist-induced activation of PKCε is measured by its phosphorylation on ser729. PKCε negatively regulates ADP-induced Erk1/2 phosphorylation and subsequent thromboxane generation but contributes little to PAR4- and GPVI-mediated platelet responses. Studies of FeCl3-induced vessel injury in PKCe in PKCε knockout mice have shown that PKCε positively regulates thrombus formation (Bynagari-Settipalli et al. 2012).

PKCη positively regulates ADP-induced aggregation and thromboxane generation. Its activation is measured by phosphorylation on Thr512. Platelet integrin receptor activation dephosphorylates thr512 on PKCη in a PP1γ phosphatase (a serine/threonine phosphatase) dependent manner (Bynagari et al. 2009). The role of novel PKC isoforms is depicted in Fig. 6.

Atypical PKC Isoforms PKCζ is constitutively phosphorylated in human platelets on thr410 and thr560. Thr410, but not thr560, becomes dephosphorylated upon agonist stimulation in an integrin and serine/threonine phosphatase (PP1 and PP2A) dependent manner (Mayanglambam et al. 2011). The exact role of PKCζ and its mechanism of regulation in platelet function are yet to be determined. Table 2 presents some of the platelet functions affected in the absence of PKC isoforms.

PKC isoforms also regulate each other. In cardiomyocytes, PKCε and PKCδ cross phosphorylate each other on their activation loop and hydrophobic domain, respectively, and regulate their activities (Rybin et al. 2003). In platelets, classical PKC isoforms regulate Y311 phosphorylation on PKCδ and subsequent thromboxane generation downstream of ADP receptors possibly through a phosphatase and a tyrosine kinase (Bhavanasi et al. 2015). PKC substrates in platelets that were known to regulate secretion include alanine-rich C-kinase substrate (MARCKs) (Hartwig et al. 1992), platelet leukocyte C kinase substrate (Pleckstrin) (Nishizuka 1988; Tyers et al. 1989), and cytohesin-2 are known to be PKC substrates in platelets that regulate platelet secretion (Elzagallaai et al. 2000) (Lian et al. 2009) (van den Bosch et al. 2014). Identifying and targeting isoform-specific substrates could be beneficial in developing antithrombotic drugs as PKC isoforms could be differentially regulating platelet functions through isoform-specific substrates.

PKC isoforms play an indispensable role in regulating various functions such as thrombosis, heart failure, ischemia–reperfusion injury, metastasis, cell division, development, apoptosis, etc. (Bynagari-Settipalli et al. 2010; Koivunen et al. 2006; Reyland 2007), hence, caution must be exercised while using these PKC inhibitors to treat a specific disease as they might affect other functions of the body. Phosphorylation-specific inhibitors of PKC will hopefully help keep any undesirable side effects under check as compounds that inhibit only specific functions elicited by PKC isoforms could be used based on the disease treated. Currently available inhibitors of PKC are mostly ATP competitive inhibiting the ability of PKC isoforms to phosphorylate their downstream substrates and subsequent signaling responses. However, not all functions of PKC isoforms could be attributed to their catalytic activity as PKC isoforms also exhibit kinase-independent functions in various cells possibly by acting as scaffolds (Goerke et al. 2002; Kim et al. 2012). Hence inhibitors have to be designed in a way that they bind to different domains of PKC isoforms to identify the role played by individual domains of PKC isoforms. Also, inhibitors that inhibit/dissociate interactions between PKC isoforms and different molecules must be designed and studied for their impact in regulating cell functions.

Protein Kinase D Protein kinase D2 (PKD2) is the major isoform present in platelets and gets activated by phosphorylation on ser744 and ser748. It positively regulates agonist-induced platelet dense granule secretion and thrombus formation on collagen under flow (Konopatskaya et al. 2009). PKD2 is also phosphorylated on ser916, an auto-

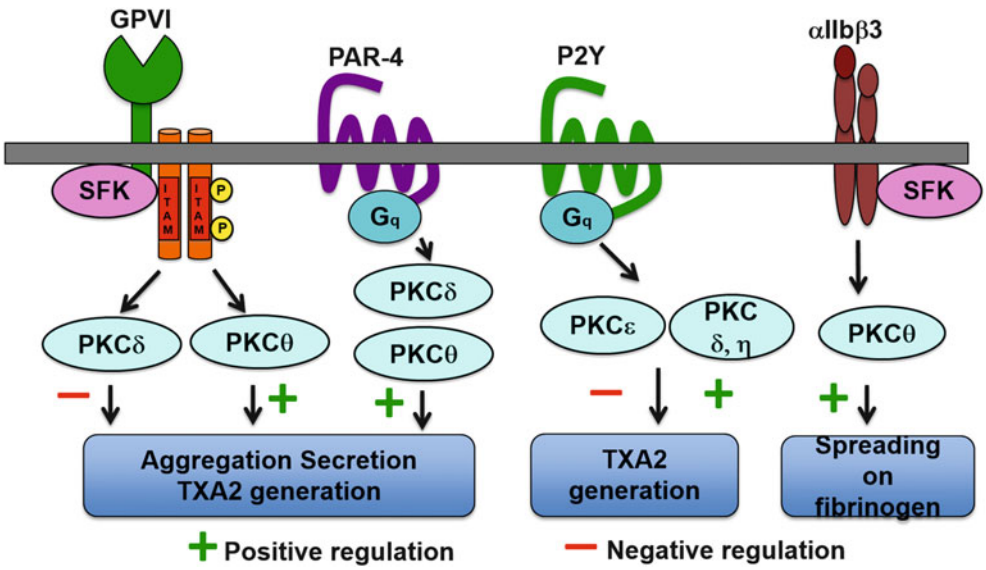

Fig. 6 Role of novel PKC isoforms in regulating platelet activation

Table 2 In vivo and in vitro roles played by classical and novel PKC isoforms

Ablation of PKC isoform	Spreading on fibrinogen	Thrombus formation on collagen	In vivo thrombus formation: time to occlusion	Bleeding times
PKCα	No defect (Konopatskaya et al. 2009)	Inhibition (Gilio et al. 2010)	Increased[a] (Konopatskaya et al. 2009)	No defect (Konopatskaya et al. 2009)
PKCβ	Inhibition (Buensuceso et al. 2005)	Inhibition (Gilio et al. 2010)	XX	XX
PKCδ	XX	Potentiation (Gilio et al. 2010)	No defect[b] (Chari et al. 2009a)	XX
PKCθ	Inhibition (Soriani et al. 2006)	Potentiation (Gilio et al. 2010; Hall et al. 2008)	Increased[b] (Nagy et al. 2009)	Increased bleeding times (Cohen et al. 2009)
PKCε	No defect (Pears et al. 2008)	No defect (Pears et al. 2008)	Decreased[b] (Bynagari-Settipalli et al. 2012)	Decreased bleeding time (Bynagari-Settipalli et al. 2012)
PKCη	XX	XX	XX	XX

[a]Laser-induced injury
[b]FeCl3-induced injury model
XX data not available

phosphorylation site by classical PKC isoform α (Konopatskaya et al. 2009), and on ser744 and ser748 by novel PKC isoform δ (Bhavanasi et al. 2011) in platelets.

Protein Kinase G and Protein Kinase A In healthy blood vessels, the endothelium releases nitric oxide and increases cGMP and subsequently cAMP which activate Protein kinase G (PKG) and Protein kinase A (PKA), respectively. These kinases restrict abnormal platelet activation in healthy blood vessels through Vasodilator-stimulated phosphoprotein (VASP) (Haslam et al. 1999; Jensen et al. 2004). VASP negatively regulates platelet function and is a major substrate for PKA and PKG but can also be phosphorylated by PKC. It gets phosphorylated on ser157, ser239, and thr278 (Butt et al. 1994) (Chitaley et al. 2004; Wentworth et al. 2006). PKG was also shown to regulate platelet activation in a biphasic manner consisting of initial stimulatory effect and subsequent inhibitory response (Li et al. 2003). Upon activation, PKA inhibits Rac signaling (Gratacap et al. 2001) and phosphorylation by PKA on thr203 of Gα13 inhibits Rho signaling leading to platelet aggregation (Manganello et al. 2003).

Casein Kinase Casein kinase (CK), another ser/thr kinase has been shown to positively regulate platelet activation including aggregation, secretion, and thromboxane generation. It phosphorylates phosphatase and tensin homolog (PTEN) on ser380, which inactivates PTEN thereby leading to the PI-3 kinase signaling required for platelet activation (Ryu and Kim 2013).

Akt/Protein Kinase B Akt is a 57-kDa serine/threonine protein kinase that is activated via membrane binding of its

PH domain to the phosphatidylinositol 3-kinase (PI3K) products phosphatidylinositol-3,4-bisphosphate (PIP2) and PIP3. As such, membrane translocation of Akt is critical for its activation. Once at the membrane Akt is phosphorylated on threonine 308 via phosphoinositide-dependent kinase 1 (PDK1) and on serine 473 via mammalian target of rapamycin complex 2 (mTORC2). The contribution of each phosphorylation site to the activation of Akt and subsequent effects on platelet reactivity are unknown but worthy of investigation.

There are three isoforms of Akt (Akt1, Akt2, Akt3), all of which are expressed in mouse and human platelets. In mouse platelets, Akt3 appears to be the most highly expressed isoform, but the relative expression of each isoform in human platelets is unknown (O'Brien et al. 2012). It is apparent that each Akt isoform has an independent function, but there is also a good deal of overlap between the isoforms. For instance, deletion of each Akt isoform restricts platelet response to thrombin (Chen et al. 2004; Woulfe et al. 2004; O'Brien et al. 2011). However, only deletion of Akt1 seems to effect collagen-induced platelet activation (O'Brien et al. 2011). Furthermore, Akt1 and Akt3 regulate $\alpha_{IIb}\beta_3$-dependent signaling, but Akt2 is dispensable (O'Brien et al. 2011).

In platelets, Akt is activated by a number of different agonists. However, in each case, Akt activation is dependent on secretion of ADP from dense granules and subsequent activation of the $P2Y_{12}$ receptor. Blockade of the $P2Y_{12}$ receptor using the antagonist ARC69931MX greatly reduces phosphorylation of Akt regardless of the agonist used (Gratacap et al. 2011; Kim et al. 2004, 2009; Cipolla et al. 2013).

Interestingly, translocation of Akt to the membrane, which is necessary for Akt activation, may be mediated by G_q-coupled protease-activated receptors (PAR) (Badolia et al. 2015). Phosphorylation of Akt is greater in human platelets activated with PAR agonists than those activated with ADP. Additionally, Akt translocation is also greater downstream of G_q-coupled receptors. However, inhibition of $P2Y_{12}$ eliminates Akt phosphorylation downstream of G_q while Akt translocation is unperturbed. Therefore, the translocation of Akt appears at least partially dependent upon G_q, while activation of Akt is dependent upon the G_i-coupled $P2Y_{12}$ receptor.

Akt translocation in platelets may be mediated by PAK (Badolia et al. 2015). In human platelets, Akt is constitutively associated with PAK. This association is not altered with PAK inhibition suggesting that the role of PAK is that of a scaffold. PAK has been shown to associate with GTP-bound RAC1 in thrombin-activated platelets. Inhibition of RAC1 in thrombin-stimulated human platelets largely disrupts PAK/Akt translocation to the membrane, suggesting that RAC1 is an important mediator of PAK/Akt translocation.

GSK3β Active Akt is capable of phosphorylating several targets in platelets. One such target is GSK3. Both isoforms of GSK (GSK3α and GSK3β) are expressed in platelets, but expression of GSK3β is predominant (Li et al. 2008). GSK3β is a constitutively active serine/threonine kinase that is negatively regulated by Akt phosphorylation of serine 9 (Cross et al. 1995; McManus et al. 2005). Interestingly, it appears as though Akt3 is the isoform that is predominantly responsible for GSK3β serine 9 phosphorylation (O'Brien et al. 2012). GSK3β appears to be important for platelet function as either GSK3β haploinsufficiency or inhibition causes enhanced platelet reactivity to agonist in vitro and has prothrombotic effects in vivo (Li et al. 2008). Similarly, platelets from GSK3β (as well as GSK3α) constitutively active mutant mice (GSK3α serine 21 and GSK3β serine 9 mutated to alanine) have reduced platelet aggregation and secretion in response to PAR4 agonists (Moore et al. 2013). These studies suggest that GSK3β negatively regulates platelet activity, but the exact mechanisms by which GSK3β exerts its influence are not yet known.

MAPKinases Mitogen-activated protein kinases (MAPK) are serine/threonine kinases that control a variety of cellular functions. There are 3 MAPKs expressed in platelets; p38, extracellular regulated kinase1/2 (ERK1/2), and c-Jun NH2-terminal kinase (JNK). MAPKs are activated by MAPK kinases, which are activated by MAPK kinase kinases downstream of several platelet agonists (Garcia et al. 2005, 2007; Toth-Zsamboki et al. 2003; Flevaris et al. 2009). It has been postulated that ERK1/2 activity is dispensable for primary platelet activation (Borsch-Haubold et al. 1996). However, these experiments were conducted using the MEK (MAPK kinase) inhibitor PD098059 that also inhibits cyclooxygenase and, therefore, thromboxane generation (Borsch-Haubold et al. 1998). It has since been established that ERK is an important regulator of thromboxane generation (Garcia et al. 2005; Shankar et al. 2006).

While ERK1/2 appears to regulate thromboxane generation, another MAPK, p38, appears to play no role in the initial phases of platelet activation, but may regulate outside-in signaling. Phosphorylation of p38 MAPK occurs in response to various platelet agonists (McNicol and Jackson 2003; Schwarz et al. 2000). It has been postulated that p38 is important for platelet granule release. However, the inhibitors used in these studies are known to inhibit cyclooxygenase as well as p38 (Flevaris et al. 2009). Conversely, when a specific inhibitor of p38 is utilized (VX-702) it is apparent that p38 has no role in the initial phases of platelet activation (Kuliopulos et al. 2004). Finally, p38 does appear to be important for platelet outside-in signaling (Flevaris et al. 2009).

Conclusions

Phosphorylations events play a critical role in the regulation of signals that control platelet functional responses. While certain signaling cascades have been thoroughly elucidated and their contributions to regulation of functional events have been understood, the exact role of many phosphorylations, the substrates of the kinases, and the activation and inactivation mechanisms of the kinases are still not clearly understood. Whereas many conclusions regarding the function of a kinase have been based on small molecule inhibitors and knockout mice, these conclusions have limitations. The small molecule inhibitors have nonspecific effects that become apparent as more advances are made in the field. The knockout mice might have other mechanism of regulation of signaling molecules in the progenitor cells that manifests as a platelet functional defect. Hence more studies have to be performed to unequivocally establish the role of a kinase in the signaling cascade and platelet function.

Take Home Messages

- Protein phosphorylation is the chief mechanism behind platelet activation.
- Platelets mainly express two types of surface receptors, GPCRs and Tyrosine kinase Receptors.
- Extracellular signals (via surface receptors) activate a number of intracellular proteins that are regulated by, and modulate other proteins, through phosphorylation.
- Intracellular proteins include tyrosine kinases such as Src family kinases, Syk, and Tec family kinases, as well as serine/threonine kinases like PKC, Akt, and MAPK.

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Platelet Adhesion

Elizabeth E. Gardiner and Robert K. Andrews

Abstract

Platelet adhesion is a critical functional component of blood platelets in pathophysiology and a current focus of drugs modulating platelet activity. In this chapter, we discuss platelet adhesion in terms of both basic science and clinical relevance, not only related to thrombosis and haemostasis, but also in terms of inflammation, infection, and immune-related functions of platelets. In particular, a key factor in the initiation and control of platelet adhesion in the healthy or diseased bloodstream is the effect of changes in fluid shear forces, which is also essential in control of expression and regulation of platelet-specific receptors such as glycoprotein (GP) Ib-IX-V and GPVI, and the interactions with their major binding partners, including von Willebrand factor (VWF) and collagen, respectively. It is the nature of human platelets to rapidly become adhesive to various cellular or non-cellular substrates and to secrete an array of prothrombotic and proinflammatory factors, to localize coagulation and neutrophil-mediated inflammation within the vasculature. Understanding the cellular, biochemical, and shear-related mechanisms involved in platelet adhesion can underpin new advances in the diagnosis and therapy of disease.

Introduction

Platelets are produced from megakaryocytes in the bone marrow and circulate in the human bloodstream for 7–10 days, typically at counts of around $200 \times 10^9\,L^{-1}$. Surveillance by platelets of the vasculature for injury or infection or other irregularities triggers rapid functional responses that control haemostasis, wound healing, innate immune, and inflammatory responses. Platelets also play a major role in maintaining vascular integrity, by controlling endothelial cell and barrier function. Central to these coordinated responses is the ability, within seconds to minutes, to adhere and activate, secrete from storage granules, and interact with other vascular factors and cell types. Importantly, these interactions occur even at arterial or at higher pathological flow rates and shear stresses acting against firm platelet adhesion. Some of the key platelet adhesion networks in the vasculature are shown in Fig. 1. These interactions involve: (a) platelet–platelet adhesion (aggregation), (b) platelet adhesion to the subendothelial matrix, (c) platelet adhesion to activated endothelial cells which express prothrombotic and proinflammatory factors, (d) platelet–neutrophil and platelet–monocyte interactions, and a recently discovered mechanism involving (e) glycoprotein (GP) VI-dependent platelet adhesion to polymerized fibrin formed following coagulation. These very recent latter findings are significant in providing a mechanism for GPVI-dependent platelet adhesion and activation without collagen exposure and increasing platelet-dependent thrombin generation (Mammadova-Bach et al. 2015; Alshehri et al. 2015; Chung 2015). All of these

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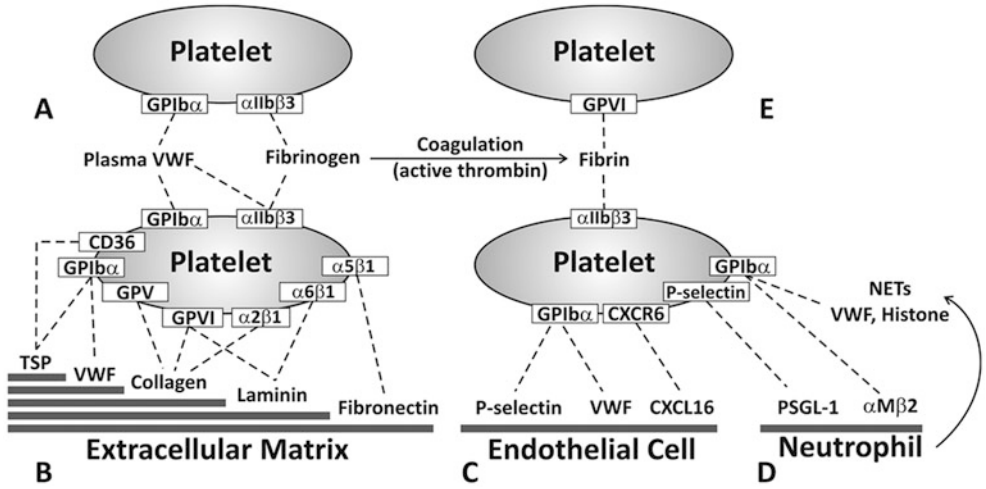

Fig. 1 Platelet adhesion networks. Platelet adhesion in the vasculature involves (**a**) platelet–platelet adhesion (aggregation) mediated by shear-dependent plasma von Willebrand factor (VWF) binding to GPIbα or activated platelet integrin αIIbβ3 binding VWF or fibrinogen, (**b**) platelet–matrix adhesion mediated by receptors on resting or activated platelets for thrombospondin (TSP), VWF, collagen, laminin, and/or fibronectin (**c**) platelet–endothelial cell adhesion mediated by GPIbα binding P-selectin/VWF on activated endothelium or as recently described in atherosclerotic conditions, chemokine-receptor interactions, or (**d**) platelet–neutrophil interactions mediated by P-selectin on activated platelets binding neutrophil P-selectin glycoprotein ligand-1 (PSGL-1), or GPIbα on unactivated platelets binding integrin αMβ2 on activated neutrophils. Platelets also potentially interact with components of DNA-based neutrophil extracellular traps (NETs) including VWF associated via histone. Platelet–monocyte interactions may involve platelet P-selectin, CD40L or GPVI binding monocyte PSGL-1, CD40, or CD147 (EMMPRIN), respectively. (**e**) Recent studies also demonstrate GPVI-dependent platelet adhesion to fibrin generated following coagulation and thrombin generation. Platelet activation/secretion pathways following adhesion are not shown. See the text for details

adhesive interactions are mediated by specialized platelet GP receptors and their counter-receptors or adhesive ligands (Fig. 1). In this regard, human platelets contain several thousand identified proteins and several thousand more predicted by platelet-expressed genes (García et al. 2004a, b; Burkhart et al. 2012). These include secreted, phosphorylated, and other groups of proteins identified by sub-proteome analysis (Senzel et al. 2009; Watson et al. 2005). Supernatants of platelets stimulated to activate metalloproteinase 'sheddases' also contain over a thousand proteins, including 69 membrane protein fragments (Fong et al. 2011). Here we will focus on the function and regulation of some of the key platelet proteins involved in platelet adhesion, focusing on clinically relevant aspects, recent developments, and future therapeutic/diagnostic opportunities.

Platelet–Platelet Adhesion (Aggregation): Introduction of Key Receptors and Ligands

The adhesion of platelets to other platelets leading to formation of clumps or aggregates in plasma or at the vessel wall is covered in detail elsewhere in this edition, but is briefly mentioned here because some of the key receptors that mediate platelet aggregation (Fig. 1a) also play an important role in other adhesive events in the vasculature, and thrombus formation is a critical event in normal haemostasis or thrombotic disease. The stability of a thrombus associated with the vessel wall is also critical in embolization and downstream thrombotic events.

In the absence of vessel wall components, platelet–platelet interactions following stimulation with soluble agonists such as thrombin or ADP are mediated by activation of a platelet-specific β3 integrin, $\alpha_{IIb}\beta_3$, which is constitutively and highly expressed on the resting platelet surface, but in an inactive conformation unable to bind plasma ligands, fibrinogen, or von Willebrand factor (VWF) (Coller 2015; Estevez 2015; Bryckaert et al. 2015). Following platelet activation leading to activation of $\alpha_{IIb}\beta_3$ and ligand-induced $\alpha_{IIb}\beta_3$-dependent signalling, binding of these multivalent adhesive ligands enables platelet–platelet aggregate formation. Exposure of platelets to high supra-physiological shear forces is also an important initiator of platelet aggregation that can occur in the absence of other external agonists or vessel wall factors (section 'Platelet Adhesion to Subendothelial Matrix'). This process is dependent upon platelet GPIbα of the GPIb-IX-V complex binding VWF in a shear-dependent manner, leading to $\alpha_{IIb}\beta_3$-dependent platelet aggregation (Kroll et al. 1996). The GPIbα-VWF interaction is exquisitely regulated, so that even though GPIb-IX-V is constitutively expressed on the platelet surface and VWF is present in normal human plasma, binding of the A1-domain of VWF to GPIbα requires either shear-dependent conformational activation of ligand, receptor, or both, enabling high-affinity interaction sustainable under shear conditions (Yago et al. 2008;

Andrews and Berndt 2008). The binding site for $\alpha_{IIb}\beta_3$ is located in a different domain (C4) of VWF (Bryckaert et al. 2015); the mature VWF subunit (~270 kDa) consists of domains D′-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK and is disulfide-linked to form high molecular weight multimers. VWF is also enabled to bind platelet GPIbα when associated with extracellular fibrillar collagen via the VWF A3 domain (section 'Platelet Adhesion to Subendothelial Matrix') or expressed as high-molecular weight multimers on endothelial cells via endothelial P-selectin (section 'Platelet-Endothelial Cell Adhesion'). The unique properties of these proteins underpin their selective interactions in health and disease.

The GPIb-IX-V complex consists of four type I transmembrane subunits that are all members of the leucine-rich repeat (LRR) family. GPIbα (610 amino acid residues; ~135 kDa; ~20,000 copies/platelet) is the major ligand-binding subunit and is disulfide-linked to GPIbβ and noncovalently bound to GPIX and GPV. GPIbα, GPIbβ, and GPIX are minimally required for surface expression of a functional complex. The ligand-binding N-terminal domain (amino acid residues 1-282) of GPIbα is composed of seven tandem leucine-rich repeats and flanking sequences and contains the binding site for VWF and other ligands (Andrews and Berndt 2012; Gardiner and Andrews 2014a), as discussed in following sections. Within the leucine-rich repeat domain of GPIbα, repeats 2–4 (residues 60–128) play a crucial role in regulating adhesion to VWF under shear conditions (Shen et al. 2000, 2006).

The cytoplasmic tail of GPIbα (~96 residues) contains defined binding sites for filamin (actin-binding protein) of the cytoskeletal actin infrastructure (Cranmer et al. 2011) and for signalling or regulatory proteins, including 14-3-3ζ and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Mu et al. 2008, 2010). The cytoplasmic domain of GPIbβ (~34 residues) also contains a binding site for calmodulin, which controls receptor expression and function (Andrews et al. 2001), and a protein kinase A (PKA)-dependent phosphorylation site (Ser166), which controls binding of 14-3-3ζ to the complex (Andrews et al. 2007). Together, these cytoplasmic interactions control normal receptor expression, VWF binding and signalling by GPIb-IX-V. GPV (544 residues, ~82 kDa) contains fifteen LRR domains and mediates platelet adhesion to collagen (Moog et al. 2001). Interestingly, like GPV, the primary platelet collagen receptor GPVI, also forms a noncovalent complex with GPIbα (Arthur et al. 2005). GPV may therefore play a role in regulating GPVI association with GPIb-IX or in regulating binding of thrombin and/or other ligands to GPIbα (De Candia 2012). In this regard, GPIbα acts as a cofactor for thrombin-dependent activation of protease-activated receptor-1 (PAR-1).

One important mechanism regulating surface expression of GPIbα, GPV, and GPVI is metalloproteinase-mediated ectodomain shedding, resulting in the irreversible proteolytic removal of the extracellular ligand-binding domain (Gardiner and Andrews 2014b). Ectodomain shedding of platelet GPIbα near to the outer surface, but N-terminal of the disulfide bond linking GPIbα to GPIbβ, generates a soluble ~130-kDa fragment termed glycocalicin and a membrane-associated remnant fragment that remains bound to GPIbβ/GPIX. GPIbα shedding on human platelets can be mediated by the surface-expressed metalloproteinase, ADAM-17 (Gardiner et al. 2007). Ectodomain shedding of GPV is mediated by thrombin (Berndt and Phillips 1981) or by metalloproteinases (ADAM17 or ADAM10) acting at distinct sites (Gardiner et al. 2007). Shedding of GPVI from human platelets is predominantly, if not exclusively, mediated by ADAM10 generating an ~55-kDa soluble fragment (sGPVI) containing the two extracellular immunoglobulin domains, and an ~10-kDa remnant tail, and is induced by exposure of platelets to high shear stress, activators of ADAM family metalloproteinases, calmodulin antagonists, apoptotic signals, and other triggers (Gardiner et al. 2004, 2007; Al-Tamimi et al. 2012). The cytoplasmic domain of GPVI contains sequences that bind calmodulin (Andrews et al. 2002), an interaction that maintains stable surface expression, and for Src-family kinases involved in GPVI-dependent platelet activation (Suzuki-Inoue et al. 2002). GPVI signalling involves an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic domain of its co-associated Fc receptor γ-chain (FcRγ, required for GPVI surface expression), and surface density/clustering is an important factor in adhesion and signalling following engagement of GPVI/FcRγ by multivalent ligand due to ITAM cross-talk being prerequisite for activation of Syk-dependent pathways (Chen et al. 2002; Best et al. 2003; Watson 2009; Loyau et al. 2012; Ozaki et al. 2013). Surface clustering of GPIb-IX-V regulates VWF binding (Gitz et al. 2013) and a multimeric ligand is essential for GPIbα-dependent signal transduction (Gardiner et al. 2010), while adhesion of neutrophils via integrin αMβ2 (Mac-1) to immobilized GPIbα extracellular domain is dependent upon a threshold surface density of GPIbα under experimental flow conditions (Kruss et al. 2013).

Platelet Adhesion to Subendothelial Matrix

Understanding the relative role of receptors in platelet adhesion to collagenous extracellular matrix is complicated by a number of factors (Varga-Szabo et al. 2008; Watson 2009; Nuyttens et al. 2011; Cosemans et al. 2013). These include: the possibility of both direct interactions with collagen and indirect interactions with collagen-associated proteins that can occur at different stages of adhesion (Fig. 1b); the relative expression levels and surface density of receptors for collagen and collagen-associated ligands on platelets from different

individuals; the extent of platelet activation and/or the extent to which receptors such as integrins (such as $\alpha_{IIb}\beta_3$, or members of the β1 integrin family) are in an active, ligand-binding state; the cross-regulation between different receptors, where engagement of one receptor leads to upregulation of another receptor such as an integrin (Farndale et al. 2007); the different types of collagen (in the body these include fibrillar types I, II, III, V, XI, non-fibrillar types IX, XII, XIV, XVI, XIX, short chain types VIII, X, basement membrane type IV, triple helix domain types XV, XVIII, membrane-associated types XIII, XVII, and other types VI, VII) and the presentation of different binding motifs (Farndale et al. 2007) in conjunction with the variation in the type and amount of collagen-associated proteins in different vascular beds or atherosclerotic plaque; the relative shear stress and blood flow characteristics which favour, for example, GPIb-IX-V/GPVI binding VWF/collagen at elevated shear rates and integrins or other collagen-binding proteins at lower shear; and also the wider networks between exposed subendothelial matrix and other vascular systems, including endothelial cells and neutrophils which can interact with some of the same platelet receptors (Fig. 1). These are complex but necessary questions in terms of therapeutic agents that target adhesive events and have been addressed over a long period using a range of clinical observations, experimental systems, or models.

Experimental evidence for the relative importance of different platelet receptors may be based on previous studies of human acquired or congenital defects of receptors (GPIb-IX-V, GPVI, α2β1, CD36, and others), changes in expression levels of receptors in human atherothrombotic and other disease (GPVI, α2β1) (Samaha et al. 2005; Gardiner and Andrews 2014c), available mouse knockouts and other animal models of bleeding/thrombosis (Bender et al. 2011), the availability of antibodies and other inhibitors targeting individual receptors, and studies of ex vivo interactions of human platelets with collagen or collagen-related mimetics under various flow conditions (Farndale et al. 2007; Nuyttens et al. 2011). Some important limitations in interpreting these types of studies, therefore, include the types and species of collagen used for ex vivo studies and their potentially different affinity for different receptors, collagen types and receptor binding that occurs in different vascular beds or in atherosclerotic plaque, and key differences between mouse and human platelets/GPVI. For example, important differences between species can include relative expression levels and surface density, sequence differences in the GPVI ligand-binding domain, sequence differences in the cytoplasmic domain which can potentially affect signalling efficiency (Qiao et al. 2010), as well as different (ITAM) receptor profiles where engagement of one ITAM-bearing receptor acts synergistically with other ITAM-bearing receptor to amplify signalling (Bender et al. 2013). In addition, in genetically modified mouse models related to GPVI, there may be effects of the *GP6* gene deletion on other modifier genes which control relative contribution of GPVI to platelet function (for example, by altering extracellular matrix composition) (Cheli et al. 2008). It is, naturally enough, not always clear how well-experimental systems model specific human disease (Libby 2015). In this regard, understanding the range of platelet responses to collagen is necessary for therapeutic targeting and also monitoring changes in receptors such as GPVI as markers of human disease or response to treatment. Some of the most studied interactions between specific platelet receptors and extracellular matrix components are shown in Fig. 1b.

While the precise role of endothelial matrix-platelet receptor interactions in platelet adhesion in health and disease is not entirely certain, it is apparent that platelet GPIbα-VWF and GPVI-collagen engagements are indispensable for initial adhesion of platelets and subsequent activation and aggregation as the wall shear rate increases from low physiological levels (say 150–600 s^{-1}) to higher arterial or pathological levels (say 1500–20,000 s^{-1} or higher). Interestingly, neither GPIb-IX-V nor GPVI are targeted by antithrombotic drugs in current clinical use (Gachet 2015; Metharom et al. 2015), although newer agents involving these receptor-ligand interactions are under development (section 'Therapeutic Targeting of Platelet Adhesion'). Human platelet GPVI preferentially interacts with collagen types I and III; however, in some vascular beds, higher laminin levels potentially compensate for higher levels of collagen type II for example, with less avidity for GPVI. Laminin binding to GPVI and the integrin α6β1 (Inoue et al. 2006; Ozaki et al. 2013) may have functional effects on platelets reminiscent of collagen binding to GPVI and α2β1 (and/or other collagen-binding receptors). Other studies showing that fibrillary fibronectin can support platelet adhesion under flow conditions demonstrate the involvement of multiple receptor-ligand interactions leading to thrombus formation. Platelet adhesion to fibronectin initially involves platelet α5β1 and $\alpha_{IIb}\beta_3$, with subsequent thrombus growth mediated by GPIb-IX-V, GPVI, and Toll-like receptor-4 (TLR-4), the latter potentially via a direct interaction with fibronectin (Maurer et al. 2015). Animal models of atherosclerosis also provide evidence for GPVI-mediated platelet adhesion to atherosclerotic endothelium, by a mechanism involving binding to fibronectin (Bültmann et al. 2010).

Platelet-Endothelial Cell Adhesion

Clearly, the non-adhesion of circulating platelets in the bloodstream with the intact endothelial cell lining of the blood vessel wall is required to maintain the blood flow and prevent vascular occlusion by adherent platelets or

thrombi. This necessitates quiescent platelets and endothelium; however, platelet adhesion to endothelial cells mediated by one or more receptors (Fig. 1c) may be promoted by activated endothelial cells in response to injury, infection, inflammatory response, or other causes. Endothelial cells can rapidly express long strings of highly multimerized VWF from Weibel-Palade storage granules, with high capacity to mediate platelet adhesion via platelet GPIbα (Padilla et al. 2004; López and Dong 2005). Activated endothelial cells also rapidly (within seconds to minutes) express P-selectin stored on secretory granules, and P-selectin is another counter-receptor for GPIbα (Romo et al. 1999). Under chronic inflammatory conditions, endothelial cells upregulate expression of intercellular adhesion molecule (ICAM)-1, which mediates platelet–endothelial cell interactions via fibrinogen bridging ICAM-1 and $\alpha_{IIb}\beta_3$ on activated platelets (Bombeli et al. 1998). Furthermore, as recently described under atherosclerotic conditions, chemokine–receptor interactions involving endothelial chemokine CXC motif ligand 16 (CXCL16) and platelet-expressed CXCR6 mediate platelet adhesion to the human vasculature, promoting platelet-dependent atherogenesis, with these interactions leading to platelet activation and aggregation (Meyer Dos Santos et al. 2015). Other studies of animal models of atherosclerosis also suggest GPVI-dependent platelet adhesion to endothelium, based on inhibition by GPVI deletion and/or blockade by soluble Fc-fusion protein of dimerized GPVI ectodomain (Penz et al. 2005; Bültmann et al. 2010). Platelet–endothelial cell interactions can lead to active thrombus formation, localization of active platelets secreting procoagulant and proinflammatory mediators, and also recruitment of leukocytes. These cellular interactions are also a critical factor underlying thrombotic microangiopathies, often associated with thrombocytopenia, where thrombosis in capillaries and arterioles may follow endothelial injury. An example is thrombotic thrombocytopenic purpura (TTP) featuring larger multimer size of circulating VWF, typically due to abnormal ADAMTS13 activity due to immune or other causes, which can result in increased platelet adhesion at sites of endothelial damage within arterioles/capillaries (Zhou et al. 2010; Zheng 2015). Interestingly, inhibition of platelet GPIbα–VWF adhesion has been investigated as a potential therapeutic treatment for TTP (Feys et al. 2012; Salles and Crawley 2012) and qualitative and quantitative analyses of VWF may aid in monitoring aberrant platelet activation in essential thrombocythemia (Lancellotti et al. 2015).

Platelet–Leukocyte Adhesion

Platelet–leukocyte interactions, involving both direct adhesion and indirect regulation via secreted factors and mediators, are important in health and disease (Seizer et al. 2008; Pawelski et al. 2014; Glezeva et al. 2016; Schrottmaier et al. 2015). Like platelets, polymorphonuclear leukocytes (neutrophils) are rapidly recruited at vascular sites of injury, infection, or inflammation to fulfil their role as defensive cells. Neutrophils activated by pathogenic factors or chemokines become adhesive towards other neutrophils, endothelial cells, and platelets, under conditions of low or high shear stress at inflammatory sites (Simon and Goldsmith 2002). Platelet adhesion to leukocytes is increasingly recognized as part of the proinflammatory role for platelets (Jenne et al. 2013; Rondina et al. 2013; Ed Rainger et al. 2015; Franco et al. 2015; Armstrong et al. 2015) and involves a number of adhesive receptor–ligand interactions (Fig. 1d). On resting platelets, GPIbα can act as a counter-receptor for the β2 integrin, αMβ2, on activated neutrophils (Simon et al. 2000). On resting neutrophils, P-selectin glycoprotein ligand-1 (PSGL-1) can act as a counter-receptor for P-selectin expressed on active platelets. These important P-selectin ligands—GPIbα and PSGL-1—share common structural features, including sialylated mucin-like ectodomains and an anionic sequence rich in sulfated tyrosine residues essential for binding one or more ligands (Andrews et al. 1997). Adhesion of platelets to phagocytic leukocytes may also be involved in platelet clearance in the liver or spleen. In this regard, GPIbα is constitutively shed from platelets consistent with experimental evidence that the spatial density of this receptor is critical for optimal adhesion with neutrophil αMβ2 (Kruss et al. 2013). A change of approximately one order of magnitude in GPIbα density can significantly modulate neutrophil adhesion kinetics. Evidence suggests GPIbα clustering, shedding, or changes in glycosylation/sialic acid are important regulators of platelet clearance (Gardiner and Andrews 2014b). In addition, Ashwell-Morell receptors on hepatocytes that recognize galactose and *N*-acetylgalactosamine on desialylated glycoproteins are implicated in thrombocytopenia associated with the action of desialylating *Streptococcal* neuraminidases in sepsis, and with coagulopathy (Grewal et al. 2008; Grewal 2010; Pawelski et al. 2014). Preventing platelet desialylation, for example by use of therapeutic sialidase inhibitors, may alleviate thrombocytopenia in immunothrombocytopenia associated with anti-GPIbα autoantibodies (Shao et al. 2015; Li et al. 2015).

An alternative, indirect pathway for platelet–leukocyte interactions involves the extrusion from neutrophils of extracellular traps (NETs), electrostatically charged adhesive networks comprised of DNA, associated histones, and secretory granule-derived serine proteases including neutrophil elastase and cathepsin G (Brinkmann et al. 2004). NETs may entrap and limit dispersal of pathogens, following exposure of neutrophils to pathogen-derived stimulatory factors, and may also trigger the intrinsic pathway of coagulation (Martinod and Wagner 2014). NET formation (NETosis) is induced by thrombin-activated platelets adhering to

neutrophils, by a mechanism involving platelet P-selectin and neutrophil PSGL-1, but is defective with platelets from P-selectin-deficient mice (Etulain et al. 2015). Histones also bind VWF (Ward et al. 1997), and VWF is a vital component of NETs (Grässle et al. 2014) providing the opportunity for platelet adhesion to NETs-associated histone/VWF via GPIbα (Fig. 1d). Platelets activated by thrombin or other agonists also induce significant NETosis (Etulain et al. 2015). In turn, activation of platelets leads to release of proinflammatory cytokines and growth factors leading to neutrophil activation and activation of the GPIbα-binding adhesive receptor, αMβ2 (Gardiner et al. 2012; Andrews et al. 2014a).

Finally, platelets adhere to monocytes via platelet P-selectin binding PSGL-1, CD40L binding CD40, GPVI binding CD147 (EMMPRIN), or other mechanisms (Schrottmaier et al. 2015). Importantly, platelets do not only interact with monocytes/endothelial cells at the vessel wall, but platelet-monocyte aggregates are measurable in the circulation, where altered levels are associated with cardiovascular or other disease states (Yong et al. 2011; Pawelski et al. 2014). The cross-talk between platelets, leukocytes, and endothelial cells mediated by direct adhesion or release of soluble mediators is not only integral to maintaining healthy vasculature and is clinically important across a range atherothrombotic and inflammatory disease states, but also points to the potential use of current or new antiplatelet agents targeting adhesive interactions as emerging treatment options.

Platelet–Fibrin Adhesion

GPVI-dependent platelet adhesion to polymerized fibrin formed following coagulation (Fig. 1e) is a recent finding providing a new physiological ligand for GPVI-dependent platelet adhesion and activation without the requirement for collagen or extracellular matrix exposure (Mammadova-Bach et al. 2015; Alshehri et al. 2015; Chung 2015). With some apparent similarity to fibrillar collagen, fibrin is a polymeric molecule with repetitive domains and the capacity to activate platelets by cross-linking GPVI. Fibrinogen is prevalent in normal plasma and can bind $\alpha_{IIb}\beta_3$ on activated platelets, but does not interact with GPVI or other receptors on resting human platelets. Indeed, recent studies on the role of fibrinogen in vascular processes reveal that while fibrinogen supports $\alpha_{IIb}\beta_3$-dependent platelet aggregation, polymerized fibrin is essential for platelet adhesion and occlusive thrombus formation in vivo. Mice expressing a mutant form of fibrinogen that cannot form fibrin uniquely demonstrate the importance of fibrin in preventing excessive bleeding and forming stable occlusive thrombus in a $FeCl_3$ model (Prasad et al. 2015; Litvinov and Weisel 2015). Other studies suggest fibrinogen on the surface of experimentally produced thrombi significantly limits platelet adhesion (Owaynat et al. 2015) and that purified human fibrinogen provides an anti-adhesive coating for biomaterials (Safiullin et al. 2015). However, in the case of intravascular haemolysis, extracellular haemoglobin can interact with the VWF A1 domain and promote GPIbα-dependent platelet adhesion to fibrinogen-coated surfaces, providing an unexpected adhesive mechanism linked to thrombus formation (Da et al. 2015).

Supporting a functional role for platelet GPVI–fibrin adhesion, GPVI-deficiency or anti-GPVI Fab (9O12) compromised thrombin generation and depended on fibrin polymerization, and the Fab 9O12 inhibited platelet adhesion to fibrin at low or high shear rates (Mammadova-Bach et al. 2015). In a separate study, noting that in a $FeCl_3$ injury model, GPVI-deficient mice tend to show initial platelet adhesion but not progression to stable occlusive thrombus, evidence for fibrin binding to platelet GPVI was demonstrated by linking increased tyrosine phosphorylation of GPVI/FcRγ chain and Syk in human or mouse platelets to the presence of active thrombin and fibrinogen under conditions where fibrinogen binding to $\alpha_{IIb}\beta_3$ was blocked (Alshehri et al. 2015). Platelets spread on fibrin but not fibrinogen in a GPVI-dependent manner, accompanied by increased platelet procoagulant activity. These recent studies have renewed interest in the precise location of collagen/extracellular matrix exposure and fibrin formation within thrombi in mouse in vivo models of thrombus formation or human thrombus formation ex vivo, with clear implications for new antiplatelet agents.

Other Forms of Platelet Adhesion

Although not described in detail here, various additional forms of platelet adhesion are also clinically relevant. For example, platelets interact with bacteria and other pathogens (Cox et al. 2011; Morrell et al. 2014; Andrews et al. 2014a), interact with metastatic tumour cells in the circulation (Franco et al. 2015; Li 2015), and can also come in contact with implanted biomaterials such as stents, extracorporeal membrane oxygenation (ECMO), and ventricular assist devices (Murphy et al. 2015), all of which can result in haemostatic complications due to multiple causes. Increased thrombotic or bleeding risk in these diseases clearly involves many of the same receptors that participated in other vascular adhesion events. In addition, there is a newly emerging role for the ITAM-bearing C-type lectin-like receptor on human platelets, CLEC-2, in mediating platelet adhesion to the *O*-glycosylated mucin, podoplanin, expressed on lymphatic endothelial cells and other non-vascular cell types. Platelet CLEC-2 in mice is essential for lymphatic

development as well as for maintaining lymph node stability in adults, and CLEC-2 deficiency results in abnormal lymph node development, blood-lymph mixing, and immune impairment, and for this function, there is a demonstrable requirement for platelet CLEC-2 signalling (Navarro-Núñez et al. 2013; Bénézech et al. 2014). In related model systems, defects in lymphocyte transition at lymph nodes have been observed in mice lacking podoplanin or with antibody-depleted platelet CLEC-2, as well as increased bleeding associated with immunization (Herzog et al. 2013). Recombinant podoplanin supports platelet adhesion and aggregation ex vivo under low (venous) shear conditions in a CLEC-2-dependent manner (Navarro-Núñez et al. 2015). Podoplanin may also be expressed on some tumour cells as they increase motility and migration, and CLEC-2-dependent adhesion to platelets has been implicated in tumour metastasis (Suzuki-Inoue 2011; Astarita et al. 2012).

Therapeutic Targeting of Platelet Adhesion

Antiplatelet drugs in current clinical use mainly target either platelet activation pathways (aspirin), ADP-dependent autocrine activation pathways (P2Y12 antagonists), or the adhesion receptor that mediates platelet aggregation, $\alpha_{IIb}\beta_3$ (Gachet 2015; Metharom et al. 2015). All are associated with variable increased bleeding risk in individuals, or lack of efficacy in preventing thrombosis. It is interesting that the platelet-specific receptors critical for initial platelet adhesion at elevated shear stress, GPIb-IX-V and GPVI, are not currently targeted, although various agents associated with these receptor-ligand pathways have been investigated (Deckmyn et al. 2012; Metharom et al. 2015). These receptors are only expressed on megakaryocytes or platelets. In humans, the rare defects of GPIb-IX-V (Bernard-Soulier syndrome) result in variable bleeding risk, from relatively mild to more severe (López et al. 1998; Andrews and Berndt 2013). In mice, GPIbα also appears to have a far greater contribution to stable arterial thrombosis than VWF (Bergmeier et al. 2006). Rather than completely blocking VWF binding, which would potentially increase bleeding risk, other approaches might involve selective small molecule allosteric inhibitors (Benard et al. 2008), which prevent GPIbα attaining a ligation-competent conformation via structural localization within the leucine-rich repeats and C-terminal flanking sequences (McEwan et al. 2009). Selectivity of GPIbα-targeting inhibitors for individual-binding partners is feasible, based on discriminatory inhibition of one or more ligands by monoclonal antibodies against specific sites within the ligand-binding domain (Shen et al. 2000; Andrews and Berndt 2012).

GPVI has been extensively investigated as a potential antiplatelet target (Zahid et al. 2012; Andrews et al. 2014b; Gawaz et al. 2014; Jiang and Jandrot-Perrus 2014; Jiang et al. 2015). This is due in part to expectations that the bleeding risk may be minimal, based on minimal increase in bleeding in GPVI-deficient mice (Bender et al. 2011) and the evaluation of bleeding severity in humans with congenital or acquired GPVI deficiency (Arthur et al. 2007). One GPVI-related agent is a dimeric form of soluble GPVI ectodomain expressed as a fusion with humanized Fc (Ungerer et al. 2011; Gawaz et al. 2014). Unlike native sGPVI shed from platelets which is monovalent and binds collagen weakly, the bivalent form can inhibit platelet adhesion to collagen. Testing these and other agents, including the Fab 9O12 (Mammadova-Bach et al. 2015), for blockade of GPVI-fibrin interactions is a promising future direction (section 'Platelet-Fibrin Adhesion').

Finally, in addition to targeting platelet adhesion in thrombotic disease, targeting platelet–endothelial cell and platelet–leukocyte interactions can potentially play a role in clinical treatment of inflammatory disease (Simon 2012; Gardiner et al. 2012). Selective targeting of functional sites on GPIbα for VWF, αMβ2, P-selectin, or other ligands (Fig. 1) without increasing bleeding risk may yet be possible, supporting ongoing research in this area.

Summary and Conclusions

Studies reviewed in this chapter reveal some of the diversity and underlying complexity of platelet adhesion pathways, including the diverse receptor–ligand interactions and effects of altered rheological flows, as well as the ongoing worldwide interest and accumulation of literature in this area. Clearly, targeting mechanisms of regulating receptor expression and shedding and/or intracellular signalling pathways associated with receptor engagement discussed elsewhere in this edition could ultimately provide more desirable modes of clinical targeting of platelet adhesion rather than targeting ligand binding. Nevertheless, better understanding of the precise physiological and pathological roles of platelet-specific receptor adhesive function is essential and ultimately can have a major impact on global burden of vascular diseases.

Take Home Messages

The capacity of blood platelets to rapidly adhere to numerous vascular targets underpins the important role of platelets in patho-physiology. This sheer complexity, including multiple ligands, time-dependence, and blood flows, is not only a challenge to define and understand, but also offers an abundance of new avenues to more efficaciously regulate platelet adhesion in future therapeutic applications in human disease.

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Platelet Shape Change

Joseph E. Aslan

Abstract

Platelets circulate quiescently in a flat, discoid state and undergo specific morphological changes to support granule secretion, integrin activation, platelet–platelet aggregation, adhesion, and contractile processes in the initial stages of the platelet activation program. Dynamic changes in platelet shape have long been associated with platelet activation and hemostatic and thrombotic function. Classical microscopy studies, together with the development of light transmission aggregometry, allowed for an advance of platelet shape change assays as important experimental and clinical tools. Pharmacology efforts have specified how platelet agonists, including thrombin, collagen, and ADP activate G protein-coupled receptors (GPCRs) or ITAM-bearing receptors to increase myosin light chain phosphorylation to drive actomyosin contractions and other dynamic cytoskeletal processes that form the basis for the initiation of platelet shape change. Advances in imaging technologies and molecular tools continue to provide mechanistic insights into platelet shape change as one of the earliest morphological manifestations of platelet activation, a potential biomarker for platelet-based disease states and a powerful tool in basic and translational studies of platelet function.

Introduction

As the primary cellular mediators of hemostasis and thrombosis, platelets respond to physiological cues and other stimuli with precisely regulated changes in cellular shape to support adhesion, spreading, and aggregation in the formation of hemostatic plugs and thrombi. Platelet shape change is ideally described as a transition of platelets from flat, concave discs to a more spherical form, often with pseudopodial protrusions, produced by actomyosin action, and which occur immediately following exposure to an agonist (Hartwig 2006) (Fig. 1). Shape change is considered to be the first readily measurable physiological response of platelets to a given agonist (Kim and Kunapuli 2011), detectable in the initial second of the platelet activation program (Redondo et al. 2006; Gear 1994). In physiological contexts, platelet shape change is initially reversible and can take place independently of other steps of platelet activation to support specific aspects of platelet function such as the centralization of secretory granules, granule release, the formation of filopodial structures and the aggregation of platelets with one another (Kinlough-Rathbone et al. 1976) (Fig. 2).

Platelet shape change can be broadly defined as any detectable alteration of platelet morphology, topology, surface area, volume, surface structure, contractibility, density, membrane ruffling, and blebbing (Frojmovic and Milton 1982). While platelets undergo a number of structural, morphological, and surface changes to support platelet activation, platelet biologists and hematologists historically use the term "shape change" to refer to changes in the manner in which platelets in solution scatter light, as measured by light transmission aggregometry (LTA), or Born aggregometry, in response to

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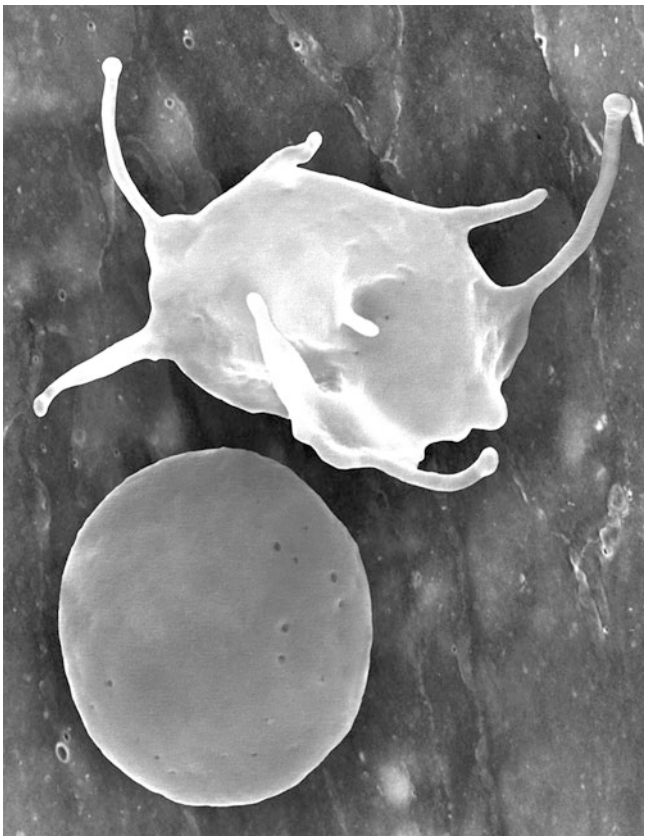

Fig. 1 Platelet shape change as visualized by electron microscopy. Electron micrograph of resting (*bottom*) and activating platelets (*top*). Quiescent platelets display a discoid morphology and undergo a change in shape to a more spherical, pseudopodia-projecting form upon activation. Copyright Dennis Kunkel Microscopy, Inc. Used with permission

Fig. 2 Platelet shape change and aggregation in solution. Electron micrograph of platelets undergoing shape change and aggregation in solution following stimulation with thrombin. Copyright Dennis Kunkel Microscopy, Inc. Used with permission

an agonist such as ADP (Rand et al. 2003). The in vitro nature of LTA, often requiring supraphysiological levels of agonist, as well as complexities brought about by changes in light transmission resulting from platelet microaggregate formation and other factors often bring the physiological relevance of shape change as studied by LTA into question (Maurer-Spurej and Devine 2001; Kitek and Breddin 1980). Nonetheless, regardless of the caveats of LTA assays, "extent of shape change" as determined by LTA continues to serve as an important, standardized, and reproducible aspect of the earliest stages of platelet activation (Holme et al. 1998).

Under conditions of physiological shear, platelet shape change allows for a dramatic increase in platelet surface area that assists in recruiting platelets into a growing hemostatic plug or thrombus, where contractile forces generated by shape change also support aggregate consolidation. In vivo, the roles and mechanisms of shape change are less straightforward, as discoid platelets in circulation reversibly change shape as they associate with a vessel wall or with the superficial layers of a growing thrombus, where platelet shape change contributes to platelet aggregate stability (Kuwahara et al. 2002; Maxwell et al. 2007; Stalker et al. 2014). The classical morphological, physiological, and practical aspects of platelet shape have been extensively reviewed (Frojmovic and Milton 1982; Aslan et al. 2012). In this chapter, we present an overview of the platelet shape change process from the perspective of classical platelet studies, current and developing measurement technologies, pharmacological manipulation, physiological regulation, and translational considerations.

Observing and Measuring Platelet Shape Change

Platelet shape change is studied using a variety of techniques to examine individual platelets or platelet populations, fixed at specific stages in the activation process or in real time in response to a given stimulus or in a specific physiological context. Methods to study platelet shape change range dramatically, from simple observations of the swirling properties of a solution of platelets by eye (Bertolini and Murphy 1994), to the latest advances in super-resolution and intravital microscopy technologies (Stalker et al. 2014). As "shape change" historically refers to the initial response of platelets to an agonist as measured by light transmission

aggregometry (LTA), assays that record changes in the light scattering properties of platelet suspensions over time are most commonly used to study shape change and platelet function (Rand et al. 2003). Such LTA studies are often complemented by microscopy analyses of platelet morphology as well as flow cytometry, volume measurement assays, and other methods that measure platelet responses (Allen et al. 1979). It is important to note that sample preparation, temperature fluctuations, and experimental conditions associated with any given shape change assay may impact platelet shape. Methods of platelet isolation, including anticoagulant choice, needle size, and blood draw procedures can also affect platelet shape (Frojmovic and Milton 1982; White 2000). The general health of human donors or model animals, as well as diet, lifestyle, medication use, and potential genetic factors should also be taken into consideration in any rigorous study of platelet morphology and shape change.

Light microscopy observations of platelets dating back to the early 1900s noted that platelets exist in a number of different morphological states and suggested that platelet shape would be associated with specific platelet functions (Ferguson 1934). In the middle of the twentieth century, electron microscopy provided additional clues into how transitions in platelet shape were associated with platelet activation, aggregation, and thrombus formation (Rodman et al. 1962). Studies of platelet shape change were advanced in the 1960s following the development of LTA, or Born aggregometry, which provided a method to follow changes in platelet shape in response to agonists in platelet-rich plasma (PRP) in real time (Michal and Born 1971; Zhou and Schmaier 2005). In LTA assays, light is passed through a glass cuvette containing a stirring suspension of platelets. The presence of platelets in solution causes the passing light to scatter such that a portion of the light is obstructed from passing through. The total light that is transmitted through the cuvette and platelet suspension is recorded and provides a measure of the optical density of the solution. Following the addition of a platelet agonist, platelets eventually clump together to form aggregates, decreasing the optical density of the solution and allowing more light to transmit through. Immediately following the addition of a platelet agonist to the solution, the aggregometry apparatus will typically record an initial, brief ~10 % decrease in light transmission (or, increase in optical density) that precedes platelet aggregation by about 10–30 s (Fig. 3). This decrease in light transmission marks the initial response of platelets to an agonist and denotes the platelet shape change. Figure 3 provides a representative, illustrative example of the shape change as measured by LTA. The degree to which optical density of a suspension of platelets will change can depend on platelet concentration, shape, size, density, suspension media, stirring speed, temperature, and other parameters. Platelet inhibitors and general pharmacological agents such as tirofiban and eptifibatide can be included in the aggregation reaction to allow for studies of shape change independent of aggregation, secretion, and other events (Phillips and Scarborough 1997; Jagroop and Mikhailidis 2008).

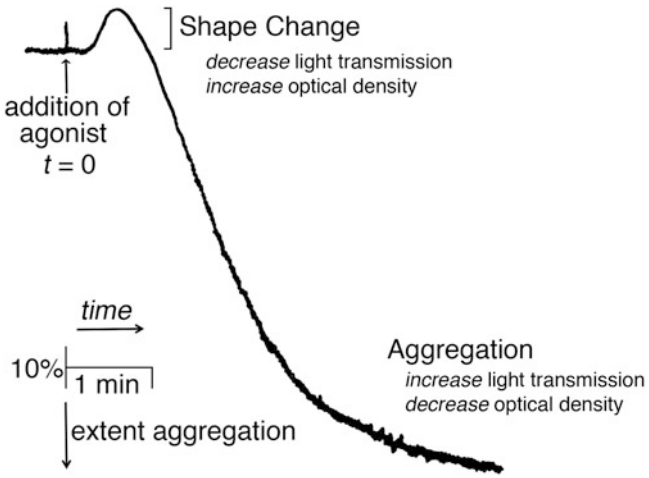

Fig. 3 Platelet shape change as determined by light transmission aggregometry (LTA). Representative aggregation trace of platelets stimulated with agonist at $t = 0$ demonstrating platelet shape change signal as a decrease in light transmission ~1 min following stimulation

In addition to LTA, more advanced light scattering methods that detect specific angles of transmitted light through a platelet solution can be used to more accurately follow platelet shape, size, and volume changes (Maurer-Spurej and Devine 2001; Mindukshev et al. 2012). While early studies of platelet shape change found a profound increase in platelet volume following activation associated with shape change (Mannucci and Sharp 1967), more recent studies suggest that shape change is associated with a small decrease in platelet volume upon activation (Moskalensky et al. 2013). The morphological aspects of shape change have been extensively detailed with a variety of microscopy methods. Atomic force microscopy has also been used to measure the contractile force of individual platelets changing shape (Lam et al. 2011). More recently, intravital microscopy has allowed for a survey of platelet morphology and intracellular signaling in vivo in the dense core as well as the more loosely packed surface of growing thrombus—revealing a complex role for shape change as platelets associate with and assemble into a stable hemostatic plug or thrombus (Stalker et al. 2014).

Maintaining Platelet Shape and Directing Shape Change

The discoid morphology of quiescent platelets is maintained by a careful homeostatic balance of intracellular signaling systems and organized structural elements which preserve

Table 1 Platelet shape change—key facts

Platelet shape change
• denotes the discoid to spheroid transition associated with platelet activation
• is the first readily detectable event of the platelet activation program
• is specifically triggered by platelet agonists
• is reversible, depending on agonist and physiological context
• is commonly observed as a small decrease in light transmission in aggregometry assays
• functionally supports hemostatic plug and thrombus consolidation in vivo
• is generally the result of actin–myosin interactions
• results from signaling events that drive a net increase in myosin light chain phosphorylation
• can occur via calcium-dependent and calcium-independent signaling processes
• has implications in platelet-associated disease states
• is associated with a loss of quality in platelet storage and transfusion

the resting state of the platelet cytoskeleton. Following stimulation, cytoskeletal dynamics support contractile, membrane, and intracellular structural changes in shape as well as secretory and adhesion events of platelet activation. Here we summarize the participation of major cytoskeletal systems in platelet shape change as well as in the maintenance of the resting platelet discoid shape. Contractile events of the actomyosin system—mediated by increases in intracellular calcium as well as calcium-independent RhoA-ROCK signaling—drive the initial stages of shape change to support secretion, aggregation, and other events of platelet activation (Bauer et al. 1999; Paul et al. 1999; Li et al. 2010). Parallel and intersecting intracellular processes promote the severing and reassembly of filamentous F-actin as well as ultrastructural changes of platelet microtubules to commit the platelet shape transition (Table 1).

Myosin Motors, Contraction, and Shape Change

Myosins are a diverse family of actin-based motor proteins that serve critical roles in cellular adhesion, migration, and contraction (Conti and Adelstein 2008). To execute these processes, nonmuscle myosins, made up of myosin heavy chain (MHC) dimers and two pairs of myosin light chains (MLCs), reversibly bind to actin filaments and hydrolyze ATP to generate mechanical force. In the initial moments of the platelet activation program, actomyosin contractions drive an isotropic contraction around the platelet perimeter, resulting in a uniform change in shape to centralize granules prior to secretion and to support other processes in the platelet activation program (Murrell et al. 2015). Myosin ATPase and contractile activity is activated by

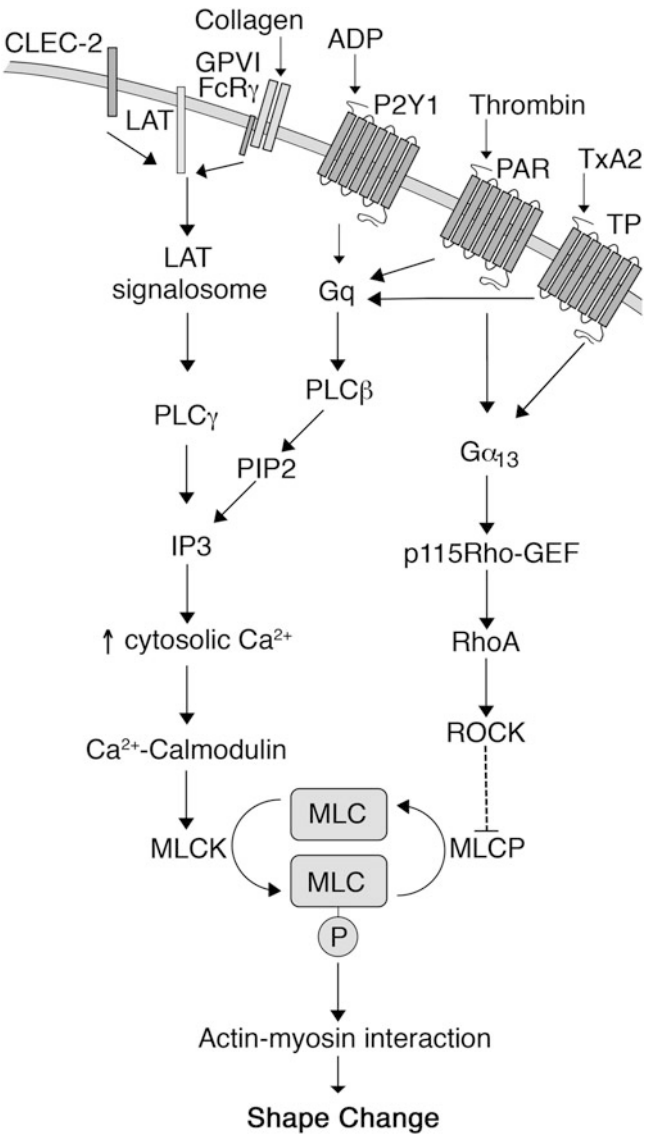

Fig. 4 Essential receptor-ligand signaling systems drive platelet shape change through calcium-dependent and calcium-independent pathways. Upon the stimulation of platelets with GPCR (ADP, Thrombin or TxA2), ITAM receptor (Collagen, ristocetin) or other ligands, PLC-based calcium signaling pathways drive the activation of MLCK or a G_{13}-initiated cascade promotes the inactivation of MLCP. Following a net increase in MLC phosphorylation, myosin associates with actin to generate the contractile basis of the platelet shape change. See text for details

phosphorylation of the myosin light chain (MLC) at Ser_{19}, which serves as a central point of regulation in the initiation of platelet shape change (Daniel et al. 1984; Nachmias et al. 1985) (Fig. 4). Accordingly, platelet shape change is regulated by a net balance of MLC phosphorylation and dephosphorylation, as controlled through calcium-dependent and calcium-independent signaling systems. Following platelet stimulation, increases in platelet cytosolic calcium promote the activation of the calcium calmodulin-

dependent MLC kinase (MLCK), which phosphorylates MLC Ser_{19} to initiate shape change. Shape change can also be triggered independently of cytosolic calcium increases through the activation of Rho kinase (ROCK), which phosphorylates and inhibits the myosin light chain phosphatase (MLCP) to result in a net increase in MLC Ser_{19} phosphorylation, contraction, and shape change.

Platelets express the nonmuscle myosin heavy chain isoform gene *MYH9*, which encodes the nonmuscle myosin heavy chain IIA. Inhibition of myosin II ATPase activity with blebbistatin—which blocks myosin–actin interactions (Kovacs et al. 2004)—inhibits platelet shape change (Johnson et al. 2007). Mouse platelets with *MYH9* deletions have impaired contractile shape change and hemostatic defects in vivo (Leon et al. 2007). Interestingly, mouse *MYH9*-null platelets have normal aggregation, secretory, and spreading capabilities, but have defective stress fiber formation associated with impaired thrombus formation under flow over collagen. Similarly, different mutations in patients with *MYH9*-related disorders are associated with a number of phenotypes—including an absence of shape change (Canobbio et al. 2005) as well as cytoskeletal abnormalities, disorders of platelet function, and platelet production (Althaus and Greinacher 2009).

Actin Filaments

Actin—the most abundant protein in platelets—comprises well over 10 % of the total platelet proteome and has central roles in platelet activation and function. In resting platelets, a network of cross-linked F-actin associates with a filamentous membrane skeleton made up of spectrin and filamin, linked to the platelet plasma membrane by the glycoprotein receptor GPIb to give structural integrity and flexibility to the characteristic discoid platelet shape (Hartwig and DeSisto 1991). Concomitant with actomyosin contractions, a complete change in platelet shape requires the disassembly of the membrane skeleton, a disruption of resting platelet actin filaments, and ultimately assembly of new actin structures characteristic of activating platelets (Hartwig 2006). Despite well-established roles for actin in filopodia formation and platelet aggregation, the roles of the actin cytoskeletal system in the initial moments of platelet activation and shape change are less clear. Agents that disrupt the actin cytoskeleton such as latrunculin A and cytochalasins prevent platelet pseudopodia formation and inhibit shape change (Patscheke and Worner 1978; Woronowicz et al. 2010; Maurer-Spurej and Devine 2001; Casella et al. 1981). Immediately following platelet stimulation, the spectrin-rich actin membrane cytoskeleton is reorganized by actin-severing proteins, including gelsolin, which interacts with, severs, and caps actin filaments to prevent elongation in a calcium-dependent manner. The severing activities of gelsolin, together with the release of the capping protein adducin from the spectrin–actin cytoskeleton help to remodel the resting platelet membrane cytoskeleton to contribute to changes in platelet shape in the platelet activation program (Hartwig 2006; Barkalow et al. 2003). Mouse platelets lacking gelsolin expression have higher resting levels of F-actin content and display hemostatic defects and inhibited shape change responses (Witke et al. 1995; Falet et al. 2000). Similarly, platelets from patients with hereditary gelsolin Asp187Asn-related amyloidosis show abnormal shape change (Kiuru et al. 2000). Studies of platelets null for the expression of cofilin, another actin-severing protein associated with shape change, suggest that cofilin does not have an essential role in shape change but contributes to later events in platelet activation (Bender et al. 2010). Platelet gelsolin activity is held in check by the vasodilator-stimulated phosphoprotein VASP, which dampens platelet activation and shape change by stabilizing actin and preventing severing by gelsolin (Bearer et al. 2000). Experiments that take advantage of blocking antibodies suggest that the actin-nucleating Arp2/3 complex may support actin remodeling events in platelet shape change (Li et al. 2002) in a manner independent of the Arp2/3 activator WASp (Falet et al. 2002; Oda et al. 2005).

Microtubules and Marginal Band Dynamics

A circumferential, marginal band of microtubules surrounds the inner periphery of discoid platelets and is one of the most notable ultrastructural features of the platelet observed by electron microscopy (White 1968). The microtubule marginal band is a major platelet structural element that confers the discoid shape of resting platelets. Microtubule depolymerization with pharmacological agents such as nocodazole and colchicine or cold treatment reversibly transitions discoid platelets to a spherical shape—a process referred to as spherocytosis (White and Rao 1998). However, microtubule stabilization with taxol does not prevent shape change, suggesting that the marginal band is important to the maintenance of resting platelet shape rather than the platelet shape change process (White and Rao 1983), especially given the rapid kinetics of shape change relative to changes in marginal band state. The marginal band is composed of 8–12 dynamic microtubules that continuously polymerize from multiple assembly sites in both directions around the circular marginal band (Patel-Hett et al. 2008). These microtubules are polymers made up of dimers of α-tubulin and β-tubulin monomers. About one half of the tubulin content in resting platelets can be found as polymerized tubulin in the microtubule marginal band (Steiner and Ikeda 1979). Nearly 90 % of the β-tubulin in

platelets is β1-tubulin, a megakaryocyte/platelet-specific β-tubulin isoform (Italiano et al. 2003). β1-tubulin content is required for marginal band integrity, as depletion, mutation, or disruption of β1-tubulin causes platelet sphering. Despite sphering, β1-tubulin-null mouse platelets preserve normal hemostatic platelet function. Interesting, Q43P mutation of β1-tubulin is associated with platelet spherocytosis as well as decreased sensitivity to platelet agonists in humans and may be thromboprotective in men (Freson et al. 2005). Microtubule-associated regulatory proteins such as RanBP10, an activator of the Ran GTPase, also associate with β1-tubulin and have roles in microtubule assembly and in maintaining platelet discoid shape (Kunert et al. 2009), and platelet shape change (Meyer et al. 2012). Genetic abnormalities in the X-linked megakaryocyte transcription factor GATA1 impact the expression of a number of megakaryocyte and platelet-specific genes, including β1-tubulin, and can alter resting platelet shape and shape change response to agonists (Hughan et al. 2005).

Studies of marginal band and microtubule dynamics have suggested a number of potential fates for the marginal band in platelet shape change. As the marginal band is not readily resolved by EM following platelet stimulation or cold treatment, it was initially hypothesized that a breakdown or depolymerization of the microtubule band supported platelet activation (White and Krivit 1967). Later EM and fluorescence microscopy studies suggested that the marginal band condenses to form a smaller, tighter ring as platelets activate and contract to change shape in the disc to sphere transition (White and Burris 1984). More recent studies that bring together fluorescence 3D confocal microcopy, pharmacological, and cell biological methods suggest a more integrated model based on the continual polymerization of the marginal band in both directions, as mediated by microtubular motor proteins kinesin and dynein (Diagouraga et al. 2014; Sadoul 2015). In this model, microtubules of the marginal band continue to polymerize as platelets activate and change shape. In the initial stages of activation and shape change—or following mild stimulation—the expanding marginal band folds in on itself into a coiled-coil microtubule structure, resembling the seam of a tennis ball. A similar marginal band morphology has been reported in platelets lacking profilin1 which have abnormally organized, hyperstable microtubules (Bender et al. 2014). Without further stimulation, this coiled-coil band eventually finds its way back to a ring shape, reverting the platelet to a discoid shape. As platelet stimulation and activation proceeds, the continuously polymerizing microtubules of the coiled-coil divert from their two-dimensional circular path to eventually form a new, flat, more compressed microtubule ring in the activated, smaller, spherical activated platelets. As tubulin polymerization continues, microtubules may eventually enter extending protrusions to support platelet filopodia formation, aggregation, and spreading. Similar microtubule twisting-based shape change mechanisms occur in nonmammalian, nucleated thrombocytes to mediate hemostasis (Lee et al. 2004).

Agonist-Receptor Systems as Initiators of Shape Change

Platelet agonists engage their respective receptors to set forth intracellular signaling cascades that drive platelet hemostatic responses, especially shape change. Following shape change, platelets release additional factors such as ADP or thromboxanes that serve a positive feedback role in amplifying platelet activation localized at sites of injury. Platelet shape change occurs rapidly in response to primary and secondary platelet agonists which signal for a rapid reorganization of the platelet cytoskeleton through calcium-dependent and calcium-independent pathways (Fig. 4).

Collagen, GPVI/FcRγ and ITAM-Based Signaling

Following vessel rupture, circulating platelets are activated by basement membrane collagen through binding to the glycoprotein receptor GPVI as well as integrin $\alpha_2\beta_1$, which supports platelet adherence to sites of injury (Clemetson and Clemetson 2001). Upon ligand binding and dimerization, GPVI recruits the Src family tyrosine kinases Fyn/Lyn to phosphorylate and couple to the FcRγ chain complex (Dutting et al. 2012). Phosphorylation of the FcRγ immunoreceptor tyrosine-based activation motif (ITAM) sets forth the activation of Syk, establishing formation of the LAT signalosome, which transduces signals for shape change in response to a variety of collagens (Jarvis et al. 2002; Nieswandt et al. 2000). Studies of collagen and GPVI in platelet shape change make use of GPVI-specific agonists, notably collagen-related peptide (CRP) and GPVI-dimerizing antibodies as well as genetic models that specify roles for GPVI together with FcRγ chain complex in the shape change initiated by collagen (Nieswandt and Watson 2003; Nieswandt et al. 2001). Roles for ITAM-bearing proteins in platelet shape change and platelet function have also been advanced by studies of the snake venom toxin rhodocytin, which triggers platelet shape change and activation through the ITAM-contain ing C-type lectin receptor CLEC-2 (Suzuki-Inoue et al. 2011).

Thrombin and PARs

Following shape change, granule secretion, and the exposure of phosphatidylserine (PS) on the outer leaflet of the plasma

membrane, platelets provide a surface for the generation of thrombin (Bevers et al. 1982), a multifunctional serine protease locally generated at sites of vascular injury which serves as a potent platelet agonist as well as the primary effector protease of the coagulation cascade (Stalker et al. 2014; Monroe et al. 2002). Thrombin activates platelets via the proteolytic cleavage of protease-activated receptors (PARs), G protein-coupled receptors (GPCRs) coupled to G_q and $G_{12/13}$ to trigger shape change, as well as the secretion of secondary platelet activators (ADP, TxA2, serotonin) that further potentiate platelet shape change and localized platelet activation (Coughlin 2000). Of the four PAR family members, PAR1 and PAR4 are expressed by human platelets; mouse platelets express PAR3 and PAR4. PAR1 responds to low-dose thrombin while higher concentrations are needed for PAR4 stimulation. Studies of thrombin and PARs in platelet shape change have been facilitated by selective PAR agonist peptides as well as mouse models of PAR deletions and other signaling mediators downstream of PARs (Sambrano et al. 2001; Wu et al. 2002). For example, the PAR-1 agonist peptide TRAP-6 (SFLLRN) promotes platelet shape change and full platelet activation of human platelets while the PAR-1 interacting peptide YFLLRNP selectively stimulates PAR-1 to elicit shape change independent of calcium mobilization (Rasmussen et al. 1993; Otterdal et al. 2001).

ADP, ATP and the Purinergic P2 Receptors

The platelet purinergic P2 receptors $P2Y_1$, $P2Y_{12}$, $P2X_1$ bind to nucleotides, notably ADP as well as ATP to support platelet activation (Kahner et al. 2006). ADP plays a critical role in platelet activation, hemostasis, and thrombus progression (Born 1985). On its own, ADP is a relatively mild platelet agonist, inducing reversible shape change and weak aggregation; however, the release of ADP from platelets serves an important role in amplifying and reinforcing platelet responses to and within a growing thrombus. After binding to ADP, $P2Y_1$, a G_q-coupled GPCR, promotes the release of calcium from internal stores to result in platelet shape change (Moers et al. 2004). Selective agonist, pharmacological inhibitor and genetic studies have confirmed that $P2Y_1$ is required for calcium mobilization and platelet shape change in response to ADP. ADP also binds to the G_i-coupled GPCR $P2Y_{12}$, well known as the target of clopidogrel and other antiplatelet agents, which couples ADP binding to $G\alpha_i$ activation to inhibit adenylyl cyclase activity, cAMP production, and PKA activation, which serves an inhibitory role in platelet activation and shape change (Offermanns 2006). While the combined efforts of $P2Y_1$ and $P2Y_{12}$ are required for complete platelet activation and aggregation in response to ADP, $P2Y_{12}$ is not required for shape change; $P2Y_{12}$-null platelets undergo shape change but have impaired aggregation in response to ADP. A third platelet purinergic receptor, $P2X_1$, is a ligand-gated, receptor-operated calcium channel that allows for the influx of extracellular calcium into platelets in response to ATP binding. Stimulation of platelets with $P2X_1$-specific agonists leads to increases in cytosolic platelet calcium and reversible shape change (Rolf et al. 2001). Like other P2 receptors, $P2X_1$ has roles in amplifying signals of platelet activation in combination with signals from other agonists and receptors such as collagen and shear-induced aggregation (Varga-Szabo et al. 2009).

Thromboxanes, TP, and Other Prostanoid Receptors

Platelets synthesize and release thromboxane (TxA2) to serve as a short-lived, locally restricted positive feedback activator of platelets (Offermanns 2006). TxA2 binds to the thromboxane receptor TP, a prostanoid GPCR coupled to G_q and G_{13}, resulting in shape change and platelet recruitment to a growing hemostatic plug. In vitro studies of TxA2 take advantage of U46619, a stable, synthetic TP agonist. In vivo, TxA2 is produced from arachidonic acid by COX-1, a well-known target of low-dose aspirin, and thromboxane synthase. Accordingly, aspirin inhibits shape change and aggregation in response to arachidonic acid and is an effective agent in the prevention of arterial thrombosis; however, aspirinated platelets maintain hemostatic functionality and maintain shape change response to a variety of other agonists (Dejana et al. 1980).

Other prostanoid receptors mediate inhibitory effects on platelet shape change and activation (Breyer et al. 2001). Prostacyclin (PGI2) and prostaglandin E1 (PGE1) bind to their own prostanoid GPCRs to increase adenylyl cyclase and cAMP levels in platelets, resulting in platelet inhibition (Best et al. 1977). cAMP activates AKAP-anchored PKA (Raslan et al. 2015), which phosphorylates and inhibits RhoA and RhoA-ROCK2-MLCP signaling, MLC phosphorylation, and shape change (Aburima et al. 2013). Nitric oxide (NO) inhibits platelet shape change through a similar PKA-based mechanism (Jensen et al. 2004).

Other Ligand-Receptor Systems

The activation of several other receptor-ligand systems triggers platelet shape change and shape change-related processes in platelets (Offermanns 2006). CD40 ligand, which binds to the CD40 receptor, activates platelets and shape change, offering a link between inflammation and thrombosis and a connection of platelet shape change in

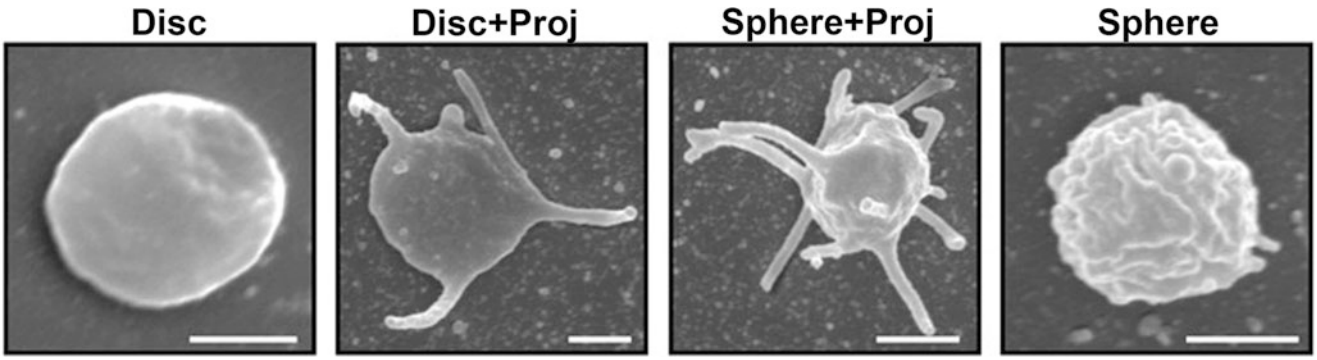

Fig. 5 Shear-dependent changes in platelet shape during translocation. Scanning electron micrographs of observed platelet morphologies during surface translocation on VWF, noting transition of disc, to disc with projections (Proj), to sphere with projections and to sphere shape. Scale bar = 1 μm. Originally published in (Maxwell et al. 2006)

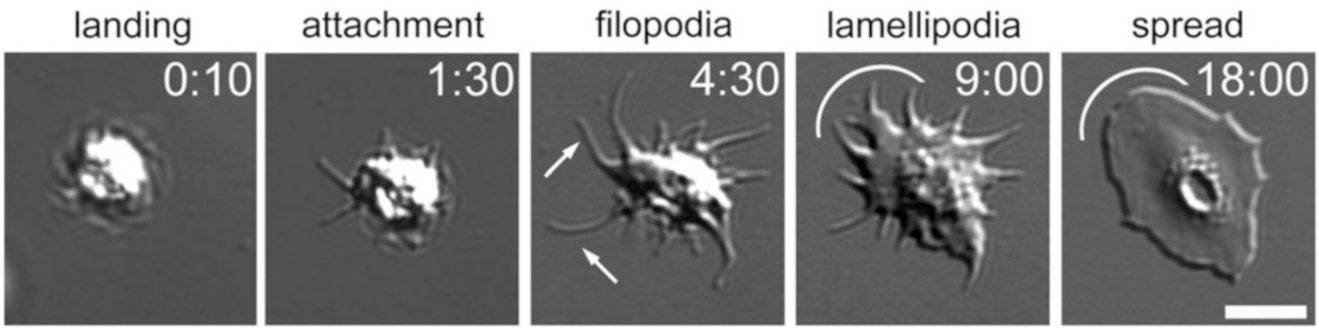

Fig. 6 Platelet shape change and spreading. Platelet attachment to and spreading on fibrinogen-coated surface visualized by differential interference contrast (DIC) microscopy. Actin-rich filopodia (*arrow*) and lamellipodia (*arc*) are indicated. Scale bar = 10 μm. Time = min:sec. Originally published in (Aslan and McCarty 2013)

inflammatory processes (Inwald et al. 2003). Serotonin binds to platelet 5HT-2_A receptors to promote shape change and to enhance the platelet hemostatic response (De Clerck and Herman 1983). Interestingly, epinephrine binds to α2A-adrenergic receptors to support weak platelet aggregation in the absence of a classical shape change (Siess et al. 1984). Interactions of platelets with VWF via the GPIb/V/IX complex cause shear-dependent, cytoskeletal-mediated changes in shape through calcium signaling which support increased rolling velocity (Fig. 5) (Mangin et al. 2003; Maxwell et al. 2006). The interaction of platelets with surface immobilized ligands such as fibrinogen, which promotes actin remodeling through activation of the integrin $\alpha_{IIb}\beta_3$, causes shape change-like processes as platelets attach to such surfaces, forming filopodial extensions and spreading through actin-rich lamellipodial sheets (Fig. 6) (Aslan and McCarty 2013). Integrin $\alpha_{IIb}\beta_3$ signaling is mediated by the ITAM-bearing platelet Fc receptor, FcγRIIa, which also transduces signals to support shape change-like platelet activation (Boylan et al. 2008). While these events are closely related to shape changes and occur in the initial steps of platelet activation by GPCRs and other receptors (Haimovich et al. 1993), critical mediators of platelet activation via integrin $\alpha_{IIb}\beta_3$, including talin (Nieswandt et al. 2007), kindlin3 (Moser et al. 2008), and Rap1 are not required for platelet shape change (Chrzanowska-Wodnicka et al. 2005).

Signaling the Platelet Shape Change

Intracellular signaling pathways downstream of platelet GPCRs, integrins, glycoprotein receptors, and ITAM-bearing receptors support specific facets of the platelet activation program, including platelet shape change (Li et al. 2010). Ultimately, these receptor signaling systems activate both calcium-dependent and calcium-independent pathways to drive changes in platelet shape through the control of myosin light chain phosphorylation and actomysosin contractions that initiate shape change (Fig. 4).

Intracellular Calcium and Phospholipase Signaling

Changes in platelet intracellular calcium concentration have long been known to signal several aspects of platelet function, including platelet shape change (Rink et al. 1982). Early studies demonstrated that the calcium ionophore A23187, which transports divalent cations across cell membranes into the cytoplasm and releases calcium from internal stores, promotes platelet shape change (White et al. 1974). Likewise, EGTA and BAPTA-AM, which chelate intracellular calcium released from intracellular stores during platelet activation, delay shape change kinetics (Bauer et al. 1999). Increases in intracellular calcium levels typically first come about through mobilization of calcium from intracellular stores, followed by another more dramatic increase in intracellular calcium store operated calcium entry (SOCE) mechanisms (Rink et al. 1982; Varga-Szabo et al. 2009). While SOCE provides the majority of cytosolic calcium increases in platelet activation, mobilization from stores alone is sufficient to activate platelets and elicit shape change.

Calcium-centric platelet activation events, including platelet shape change, are strongly tied to the activation of phospholipases PLCβ and PLCγ, which are activated by Gq and ITAM signaling, respectively. PLCs may also be regulated by PI3K/Akt to change the kinetics of intracellular platelet calcium signaling and shape change (Chen et al. 2004). PLC enzymes catalyze the conversion of the inner leaflet plasma membrane phospholipid phosphatidylinositol 4,5-trisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), which serve as second messengers in a number of intracellular signaling processes. After generation from PIP2, IP3 binds to IP3 receptors in the platelet endoplasmic reticulum (ER) membrane to promote the release of calcium from the ER. The ER, also known as the dense tubular system (DTS) in platelets, is the primary store for calcium in platelets (Gerrard et al. 1978) and serves a major role in shape changes mediated by intracellular calcium (Jardin et al. 2008). Nonetheless, the release of calcium from other organelles may contribute to platelet intracellular calcium increases (Lopez et al. 2006). Ultimately, following IP3-mediated release from intracellular stores, cytosolic calcium binds to calcium–calmodulin to activate MLCK, which then phosphorylates MLC to drive actin myosin interactions and shape change (Hathaway and Adelstein 1979) (Fig. 4). Following IP3-trigered release of calcium from the ER/DTS, store-operated extracellular calcium entry (SOCE), mediated by the intracellular calcium sensor stromal interaction molecule 1 (STIM1) and the plasma membrane channel protein Orai1 drive a major influx of extracellular calcium (Varga-Szabo et al. 2009). While SOCE has a major role in raising intracellular calcium in platelets, many aspects of platelet function, including shape change, are preserved in platelets lacking STIM1 or Ora1, suggesting that IP3-mediated release from the ER is sufficient for shape change and other functions. Given the kinetics of SOCE relative to rapid shape change and platelet activation, SOCE is more likely to support roles in thrombus stability, as shown in genetic studies of STIM1 and Ora1knockout mice (Varga-Szabo et al. 2011) and intravital studies of calcium flux in platelets in growing aggregates (Maxwell et al. 2007). Similarly, continued IP3 formation via synthesis of PIP2 by PIP5KI has roles in the stabilization of platelets in aggregates but is not required for an initial shape change (Wang et al. 2008).

RhoA-ROCK Signaling and Calcium-Independent Shape Change

The Ras homologous Rho GTPases cycle between inactive GDP-bound and active GTP-bound states to serve as signaling switches in a number of cytoskeletal process, including platelet shape change (Aslan and McCarty 2013). RhoA signaling, mediated by the Rho-activated kinase (ROCK), plays a requisite role in the calcium-independent platelet shape change downstream of G_{13}-coupled receptor activation (Pleines et al. 2012). The finding that $G\alpha_q$-null platelets fail to mobilize calcium, yet still change shape in response to certain agonists such as thrombin and U46619 ultimately allowed for the characterization of PLC- and calcium-independent mechanisms of platelet shape change (Offermanns et al. 1997). Related studies of $G\alpha_{13}$ and RhoA knockout mice as well as ROCK inhibition have likewise established that PLC- and calcium-independent platelet shape change occur through a G_{13}-RhoA-ROCK-MLCP signaling axis (Paul et al. 1999; Bauer et al. 1999).

Following the activation of G_{13}-coupled GPCRs, including TP and PARs, $G\alpha_{13}$ activates the Rho guanine nucleotide exchange factor p115RhoA-GEF, promoting RhoA-GTP formation and ROCK activation (Fig. 4) (Hart et al. 1998). Active ROCK phosphorylates and inhibits MLCP to drive a net increase in MLC phosphorylation, actomyosin contraction, and platelet shape change in a PLC- and calcium-independent manner (Watanabe et al. 2001). ROCK can also be activated through caspase cleavage in apoptosing cells (Coleman et al. 2001)—including apoptotic platelets—to promote contraction, membrane blebbing, and other cellular phenotypes common to apoptotic cells and activating platelets (Leytin 2012; Aslan and Thomas 2009). Activation of intrinsic, mitochondrial apoptotic pathways in platelets promotes shape change-like responses through ROCK in a caspase-dependent manner (Schoenwaelder et al. 2011; Kile 2014). ROCK similarly has a role in platelet

PS exposure, which also serves as a classic marker of apoptosis associated with blebbing (Dasgupta et al. 2013). Beyond the RhoA–ROCK–MLCP–MLC axis, roles for Rho GTPase family members such as Rac1 in shape change are less established. Rac1, which promotes actin polymerization through WAVE, Arp2/3, and PAK activation, plays an essential role in platelet lamellipodia formation and thrombus stabilization (McCarty et al. 2005). Interestingly, as a component of the LAT signalosome, the Rac1 GEFs Vav1/3 contributes to shape change in ITAM signaling, as discussed later (Pearce et al. 2004; Suzuki-Inoue et al. 2006). This role for Vav may in part be explained by Rac1-mediated PLCγ2 activation and calcium mobilization downstream of GPVI or CLEC-2 engagement (Pleines et al. 2009).

ITAM Signaling and Tyrosine Phosphorylation

Following phosphorylation by Src tyrosine kinases Lyn/Fyn, ITAM-bearing receptors GPVI/FcRγ and CLEC-2 activate Syk to establish the LAT signalosome, consisting of LAT, SLP-76, Vav1/3, PLCγ2, BTK/Tec, and other proteins (Suzuki-Inoue et al. 2006). Calcium mobilization and shape change in response to GPVI and CLEC-2 stimulation requires the activities of several of these signaling mediators, including Syk (Spalton et al. 2009), LAT (Pasquet et al. 1999), SLP-76 (Falet et al. 2000), Vav1/3 (Pearce et al. 2004), and BTK/Tec (Atkinson et al. 2003). The Src kinase inhibitor PP2, which prevents Syk activation downstream of GPVI or CLEC-2, also blocks shape change in response to stimulation of these ITAM-coupled receptors (Polanowska-Grabowska et al. 2003; Severin et al. 2011). Conversely, inhibitors of Src kinases can potentiate platelet shape change downstream of GPCR activation, as specific Src family members such as Fyn may serve negative feedback roles to dampen calcium signaling in response to GPCR agonists (Kim and Kunapuli 2011), suggesting a complex relationship between tyrosine phosphorylation and platelet shape change (Senis 2013; Severin et al. 2012; Senis et al. 2014).

Clinical and Translational Considerations

Platelet Storage

From a translational perspective, platelet shape change is a topic of high relevance to efforts aimed at preparing and banking platelets for transfusion. Stored platelets lose their characteristic resting discoid shape, undergoing cytoskeletal changes, forming pseudopods and shedding microparticles, characteristic of an activated phenotype referred to as the "platelet storage lesion" (Shrivastava 2009). This deleterious effect on platelet quality stems from a combination of factors as platelets remain outside of the circulation, including pH changes, mechanical stress, activation by secreted factors, and metabolic abnormalities (Maurer-Spurej and Chipperfield 2007). Moreover, platelet storage requires subphysiologcial temperatures (ideally between 20 and 24 °C) that result in calcium mobilization, actin assembly, marginal band alterations, and shape change associated with platelet activation (Hoffmeister et al. 2001, 2003; Winokur and Hartwig 1995). Understanding the molecular, metabolic, and physiological bases of shape change may allow for tools to improve the quality and shelf life of stored platelets. Advances of platelet shape assays may provide important quality controls in storage situations. Shape change-based diagnostics may also help to ensure that platelets have normal hemostatic function prior to transfusion or other clinical uses.

Platelet Shape Change in Disease

Given the early role of shape change in the platelet activation program, it is surprising that shape change is not been better developed as a diagnostic marker or therapeutic target for hemostatic and thrombotic complications associated with disease. Here we highlight instances of how shape change may provide insights into the progression, manifestation, and understanding of platelets in disease contexts. In addition to the roles of platelets in the atherothrombotic events of acute myocardial infarction (Badimon et al. 1986), platelet shape change may have roles in atherogenesis, as circulating, activated platelets may exacerbate the progression of atherosclerosis (Huo et al. 2003). In the context of cardiovascular disease, circulating platelets may become activated by oxidized low-density lipoprotein (LDL), which binds to CD36 to drive shape change and platelet activation through both calcium-dependent and calcium-independent mechanisms (Wraith et al. 2013). Atheromatous plaques can also promote platelet shape change through GPVI activation (Penz et al. 2005). Moreover, platelets from hypertensive subjects show an increased propensity to change shape via calcium signaling mechanisms (Erne et al. 1985).

Classic inherited rare platelet disorders are also associated with specific shape change phenotypes, and shape change plays an important role in the diagnosis of such disorders (Diz-Kucukkaya 2013). Platelets from patients with Glanzmann thrombasthenia lack expression of integrins and fail to aggregate but still have detectable shape change. Shape change abnormalities have been described in patients with Bernard–Soulier syndrome (Milton et al. 1985). There is also a common absence of

shape change in patients with Wilson's disease (Owen et al. 1976). Myosin serves a central role in platelet shape change, and *MYH9*-related platelet disorders provide a key example of shape change phenotypes in platelet abnormalities; individuals with a variety of different *MYH9*-related disorders have moderate bleeding tendencies associated with a loss of platelet shape change (Althaus and Greinacher 2009). As the RUNX1 transcription factor mediates the expression of MYL9, mutations in RUNX1 are associated with similar shape change phenotypes (Jalagadugula et al. 2010). Shape change abnormalities are also described in cases of Gray platelet syndrome (Nurden and Nurden 2007) and Hermansky–Pudlak syndrome (Fujimura et al. 1990).

The developing roles of platelets in a number of chronic conditions suggest that assays of shape change may serve as tools in the diagnosis, monitoring, and treatment of conditions associated with inflammation (Semple et al. 2011; Boilard et al. 2012), bacterial infection (Reddi et al. 2015; Fitzgerald et al. 2006), exposure to air pollution (Alshehri et al. 2015), and metabolic disease (Porta et al. 1980). The roles of platelets in tumor metastasis as well as the thrombotic events associated with cancer suggest a role for shape change in understanding platelet–cancer interactions (Menter et al. 2014). Differences in platelet morphology and shape change that occur with aging also provide a potential avenue for future studies.

Conclusions and Future Perspectives

Shape change represents a nearly instantaneous, readily visualized functional response of platelets to a number of stimuli in hemostatic, thrombotic, and other physiological processes. Studies of platelet shape change, through a combination of microscopy, LTA, and other methods, have provided a number of key insights into the fundamental molecular and cellular mechanisms that initiate and regulate platelet function. Through the use of pharmacological and genetic tools, a molecular definition of shape change has begun to emerge, as the phosphorylation of myosin light chain, through calcium-dependent and calcium-independent signaling routes, appears to ultimately gate the decision of platelets to change shape in the platelet activation program. However, while myosin phosphorylation has a central role in the contractile basis of platelet shape change, other cytoskeletal elements, namely, actin filaments and continuously polymerizing microtubules, can also influence platelet shape change and shape change-like behaviors through mechanisms which are less clear. Future studies of the crosstalk between myosin, actin, and tubulin cytoskeletal elements as well as their coregulation by intracellular signaling systems in platelets will help to provide a more complete molecular definition of platelet shape change in the context of the platelet activation program (Sadoul 2015).

Throughout the history of platelet studies, the majority of findings regarding platelet morphology and shape dynamics have come from static, microscopic observations or from semiquantitative LTA measurements of platelets in solution under nonphysiological conditions, defining platelet shape change from an ex vivo perspective. In vivo, studies of platelets null for the expression of key shape change mediators, including MYH9, suggest that shape change has a contractile role in thrombus consolidation and stability rather than in initiating platelet–platelet contacts and aggregation (Canobbio et al. 2005; Leon et al. 2007). A better understanding of the mechanisms and physiological relevance of platelet shape change will come about as the phenomenology of shape change is better detailed in vivo in the context of a growing hemostatic plug or thrombus where secondary platelet agonists function in specific concentration gradients to drive shape changes in aggregate incorporation and consolidation.

Despite some practical and experimental limitations, the analysis of platelet shape dynamics via light transmission aggregometry will likely remain in the platelet research and clinical laboratory toolkit for its ease of use and wide availability. However, a number of other developing, light scattering-based techniques have the potential to more readily provide greater physiologically relevant measurements of the shape change (Mindukshev et al. 2012), particularly in whole blood samples. While such technologies are not likely to replace standard light transmission aggregometry in the near future, the possibility of incorporating light scattering and other optical tools into flow cytometers and other microfluidics platforms to provide systems-level shape and size measurements of platelet populations is intriguing. In addition to light scattering-based approaches to measuring platelet shape, developing microscopy tools will always provide a powerful complement to shape change measurements, and advances in quantitative microscopy acquisition and analyses have the potential to add to data collection and statistical descriptors of resting and activating platelet populations (Kraus et al. 2010, 2014; Phillips et al. 2012). Moreover, as intravital and super-resolution microscopy technologies develop, it will be more readily possible to observe and measure changes in platelet morphology in vivo in the circulation as platelets interact with the superficial layers of a growing thrombus or with a plaque-laden arterial wall. Ultimately, similar approaches will also provide a more coherent, physiologically relevant role for platelet morphological dynamics in classical hemostatic and thrombotic contexts as well as oncological, inflammatory, metabolic, and other disease states.

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The Migration of Platelets and their Interaction with Other Migrating Cells

Eleonora Petito, Stefania Momi, and Paolo Gresele

Abstract

Platelets, beyond their well-described role in haemostasis and thrombosis, act as inflammatory cells playing an active role in several inflammatory conditions. As observed with other inflammatory cells platelets can migrate in vitro, either randomly or in the direction of a chemotactic agent, and in vivo, into inflammed tissues in response to different stimuli. In this chapter we will summarize the current knowledge about the mechanisms that regulate platelet chemotaxis, the evidence for the ability of platelets to migrate in vitro and in vivo, and the mechanisms by which platelets influence chemotaxis of other cells.

Introduction

It is now well established that platelets act as inflammatory cells and contribute to both innate and adaptive immune response through several mechanisms, like pathogen binding, trapping and killing, direct modulation of leukocyte and endothelial cell activation, leukocyte recruitment, and activation of antigen presenting cells (APC) (Czapiga et al. 2004; Jenne et al. 2013; Semple et al. 2011; Vieira-de-Abreu et al. 2012) (also see Slaba and Kubes 2017). Platelets are therefore the most abundant circulating cell type (150–400,000/μL) with an immune function and participate in host defence against parasites, bacteria and viruses. Moreover, increasing evidence shows that platelets play a pathogenic role in several chronic inflammatory disorders including atherosclerosis, allergic inflammation (asthma, rhinitis and eczema), chronic obstructive pulmonary disease, rheumatoid arthritis and inflammatory bowel disease.

Several structural and biochemical characteristics allow platelets to act as inflammatory cells (Heijnen and Korporaal 2017; Slaba and Kubes 2017), probably because they retain some functions of their phylogenetic ancestor, the amoebocyte, the unique nucleated cell with defensive and haemostatic functions circulating in the haemolymph of invertebrates (Momi and Wiwanitkit 2017).

One of the crucial functions of "bona fide" inflammatory cells is their ability to migrate through tissues. Platelets display a number of attributes compatible with the ability to migrate: they express receptors for adhesive proteins and chemokines, contain and release matrix metalloproteinases (MMPs) required for extracellular matrix (ECM) degradation, and have the cytoskeletal and enzymatic machinery required for cell locomotion.

In this chapter, we will summarize the in vitro and in vivo evidence of platelet migration in response to chemotactic stimuli and of the role of platelets in tissue recruitment of other cells, including leukocytes and cancer cells.

Chemotaxis is the active movement, or migration, of a cell in the direction of a chemotactic gradient. It is a central event in several physiologic processes, such as embryonic development, tissue repair, angiogenesis and immune response, and the abnormal chemotaxis of the cells contributes to many pathologic conditions, like chronic inflammation, autoimmunity and metastasis. Cell locomotion is a complex and multistep process by which an extracellular chemotactic gradient is detected by a specific cell receptor, a signal is translated to the cell's

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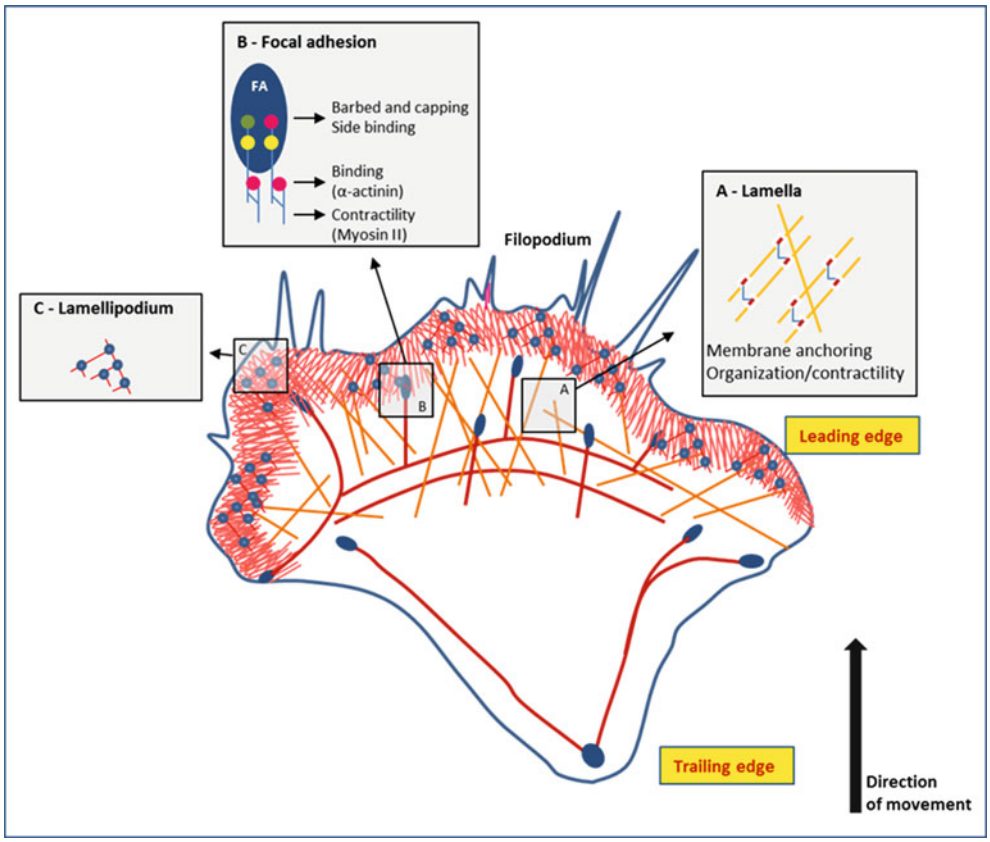

Fig. 1 Cellular polarization. Structural and functional asymmetry of a migrating cell consisting of a leading edge at the front in the direction of movement and a trailing edge at the rear associated with a cytoskeletal organization

motile apparatus and an intracellular functional and structural asymmetry is generated allowing the cell to move towards the detected chemoattractant. Cell polarization is crucial for chemotaxis and consists of the formation of two cellular compartments, the leading edge at the front and the trailing edge at the rear. At the leading edge the cell extends a protrusion, a lamellipodium or filopodium, in the direction of the chemotactic stimulus, which establishes new adhesion sites with the substratum, while at the trailing edge the cell contracts, adhesion sites detach and the uropod, a protrusion at the rear of the cell, retracts. In each of these steps several proteins and intracellular signaling pathways are involved and a fundamental role is played by the cytoskeleton and its ability to rapidly assemble and disassemble (Fig. 1) (Charest and Firtel 2007; Germena and Hirsch 2013; Jin 2013; Raftopoulou and Hall 2004).

Structural Characteristics Ascribing to Platelets the Ability to Migrate

Platelets possess all the structural characteristics required for chemotaxis. They express on their surface several functional chemokine receptors, such as CCR1, CCR3, CCR4, CXCR1, CXCR4, CXCR6, CXCR7 and CX3CR1 (Abi-Younes et al. 2000, 2001; Borst et al. 2012; Chatterjee et al. 2014; Clemetson et al. 2000; Kowalska et al. 1999, 2000; Postea et al. 2012; Rath et al. 2014; Schafer et al. 2004; Suttitanamongkol and Gear 2001; Wang et al. 1998) and other receptors involved in leukocyte migration, like Toll-like receptor 4 (TLR4) and formyl peptide receptors (FPR) (Andonegui et al. 2005; Cognasse et al. 2005; Czapiga et al. 2005).

Platelets possess the central components of chemotaxis-related intracellular signaling, and in particular phosphatidylinositol 3-kinase (PI3K) and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) that regulate the production and cellular localization of phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is crucial for the generation and maintenance of cell polarity (Van Keymeulen et al. 2006), and the Rho family of small GTPases that activate a plethora of effector molecules modulating actin cytoskeleton dynamics (Germena and Hirsch 2013; Worthylake and Burridge 2001; Yan and Jin 2012).

Platelets contain a cytoskeletal framework that allows cell movement. The discoid shape of platelets is maintained by a membrane skeleton that coats internally the plasma membrane, composed by a network of actin filaments, spectrin, adducin, and actin-associated proteins, and by a rigid cytoplasmic scaffold made of actin and filamin A. Actin-associated proteins and filamin A link the platelet cytoskeleton to integrins. Platelet activation induces changes

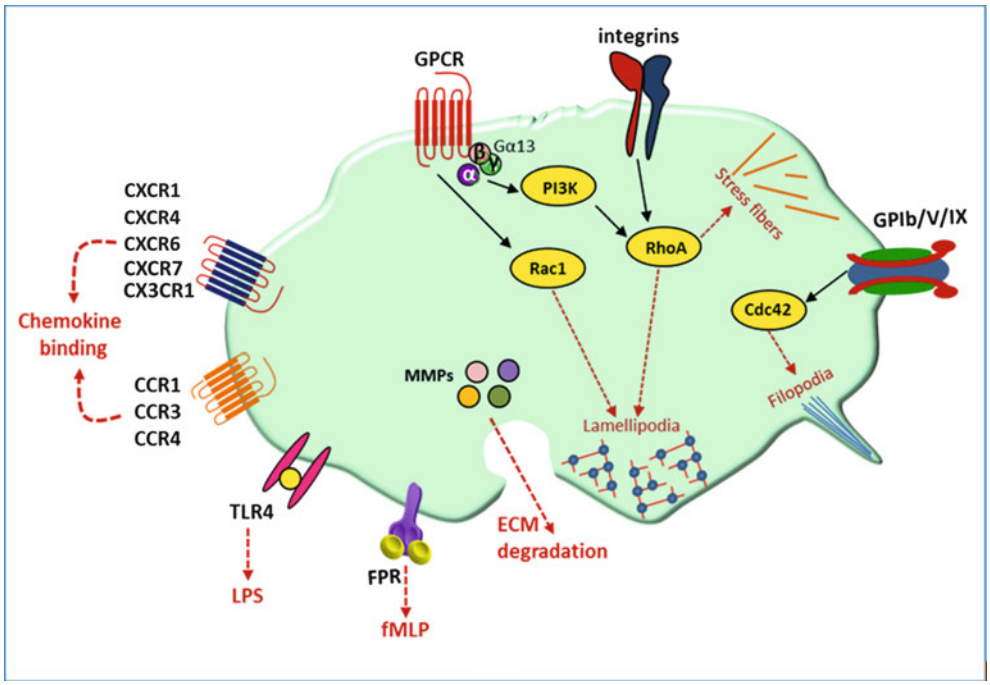

Fig. 2 Structural characteristics ascribing to platelets the ability to migrate. Platelets express on their surface several receptors triggering chemotaxis. Platelets possess the main components of chemotaxis-related intracellular signalling, involved in cell polarization and cytoskeletal re-organization. Platelets contain and release upon activation different MMPs involved in the ECM degradation required for their passage through the basement membrane

in the cytoskeletal organization with the formation of focal adhesion complexes, dynamic structures linking integrins to the actin cytoskeleton and which together with stress fibres contribute to a contractile response (Goggs et al. 2015; Hartwig 2006).

Platelets contain, and release upon activation, several MMPs, including MMP-1, -2, -3, and -14 (Seizer and May 2013; Busti et al. 2010), which may accomplish the extracellular matrix degradation required for the passage of migrating cells through the basement membrane (Fig. 2).

Platelet Migration: Studies In Vitro

The first in vitro observations on the ability of platelets to migrate date back to the early 70s. Before then, platelets were considered cell fragments passively drifting in the circulation until a contact with an area of damaged endothelium stopped them. The motion of platelets occasionally observed under a light microscope was considered as passive diffusion or Brownian movements, i.e. a temperature-dependent, erratic, not directional movement of particles smaller than 4–5 μm observed in colloidal suspensions (Chamot and Mason 1947).

The first studies on platelet migration in vitro assessed the optimal conditions to study platelet movement showing that several factors, such as temperature, pH, anticoagulant, platelet concentration and buffer composition, influence this process (Lowenhaupt et al. 1977; Nathan 1973; Valone et al. 1974) (Table 1).

Methods

Lowenhaupt and Valone were the first to study platelet migration (Lowenhaupt et al. 1973, 1977; Lowenhaupt 1978; Valone et al. 1974). Lowenhaupt adapted the capillary tube migration chamber previously described by George and Vaughan for the study of macrophage migration (George and Vaughan 1962). This consisted of an incubation chamber with a capillary tube immobilized at the bottom. The incubation chamber was composed of a stainless steel slide (75 × 25 × 3 mm) with a center hole 20 mm in diameter and one side sealed by a siliconized glass slide to form a dish and two small channels connecting it to the edge of the slide (Fig. 3a). A siliconized micro-hematocrit capillary tube, fire-sealed at one end, was filled with platelet-rich plasma (PRP) (300,000 platelets/μL) and centrifuged for 5 min. The capillary tube was then cut at the meniscus between platelet poor plasma (PPP) and the platelet pellet and secured to the bottom of the incubation chamber. The chamber was then filled with autologous PPP, covered with a siliconized cover glass and incubated at 22 °C for 18 h in a CO_2 incubator in order to maintain pH between 7.2 and 7.4. To investigate platelet chemotaxis, in the same incubation chamber, a thread-like piece of collagen or a fine-collagen-packed capillary tube was placed at a distance of about 5–6 mm from the platelet-packed capillary tube. The end point was the area of platelet migration out of the capillary tube visible by a stereomicroscope and measured with a planimeter (Lowenhaupt et al. 1973).

Table 1 Methods used to study platelet migration in vitro and optimal experimental conditions

Parameter	Optimal condition	Method	References
Temperature (°C)	22	Capillary tube[a,b]	Lowenhaupt et al. (1973, 1977, 1982) and Lowenhaupt (1978)
		7-compartment chamber[a]	
	25	Capillary tube[b]	Duquesnoy et al. (1975)
	30	Capillary tube[b]	Nathan (1973)
	37	Boyden chamber[b]	Valone et al. (1974)
		Transwell migration and videomicroscopy[a]	Czapiga et al. (2005)
		Boyden chamber[a]	Pitchford et al. (2008)
		Transwell migration and videomicroscopy[a]	Kraemer et al. (2010)
pH	5–6.5	Boyden chamber[b]	Valone et al. (1974)
	7.0	Capillary tube[b]	Nathan (1973)
	7.2–7.4	Capillary tube[a,b]	Lowenhaupt et al. (1973, 1977) and Lowenhaupt (1978)
	7.4	Transwell migration and videomicroscopy[a]	Kraemer et al. 2010
Anticoagulant	Heparin	Capillary tube[b]	Nathan (1973)
	Citrate	Boyden chamber[b]	Valone et al. (1974)
		Capillary tube[b]	Duquesnoy et al. (1975)
		Boyden chamber[a]	Pitchford et al. (2008)
	3.8 % trisodium citrate dihydrate or ACD 15 % v/v	Capillary tube[a]	Lowenhaupt et al. (1973)
		Capillary tube[b]	Lowenhaupt et al. (1977)
	ACD	Transwell migration and videomicroscopy[a]	Kraemer et al. (2010)
Incubation time (hrs)	0.25	Micromaze[a]	Lowenhaupt (1978)
	1.5	Boyden chamber[a]	Pitchford et al. (2008)
	2	Transwell migration[a]	Czapiga et al. (2005)
	3	Boyden chamber[b]	Valone et al. (1974)
		7-compartment chamber[a]	Lowenhaupt (1982)
	8	Transwell migration[a]	Kraemer et al. (2010)
	12	Capillary tube[b]	Duquesnoy et al. (1975)
	18	Capillary tube[a, b]	Lowenhaupt et al. (1973, 1977) and Lowenhaupt (1978)
	24	Capillary tube[b]	Nathan 1973
Platelet suspension	300,000/μL (PRP)	Capillary tube[a,b]	Lowenhaupt et al. (1973, 1977) and Lowenhaupt (1978)
	100,000/μL (WP)	Boyden chamber[b]	Valone et al. (1974)
	^{111}In-oxine-labeled (PRP)	7-compartment chamber[a]	Lowenhaupt (1982)
	3333/μL	Transwell migration[a]	Czapiga et al. (2005)
	300,000/μL (PRP)	Boyden chamber[a]	Pitchford et al. (2008)
	2000/μL (WP)	Transwell migration[a]	Kraemer et al. (2010)
Pore size of the filter (μm)	0.4	Transwell migration[a]	Kraemer et al. (2010)
	2	Transwell migration[a]	Czapiga et al. (2005)
	3	Boyden chamber[a]	Pitchford et al. (2008)
	8	Boyden chamber[b]	Valone et al. (1974)
Endpoint of the test	Area of migration (planimeter)	Capillary tube[b]	Nathan (1973)
			Duquesnoy et al. (1975)
	Area of migration, (stereomicroscope and planimeter)	Capillary tube[a,b]	Lowenhaupt et al. (1973, 1977) and Lowenhaupt (1978)
	Microphotographs of platelet movement	Micromaze[a]	Lowenhaupt (1978)
	Radioactive counts	7-compartment chamber[a]	Lowenhaupt (1982)
	Platelet count per microscopic field		Valone et al. (1974)
			Pitchford et al. (2008)
	Platelet count in the bottom well and image sequences of platelet movement		Czapiga et al. (2005)
			Kraemer et al. (2010)

PRP platelet rich plasma, WP washed platelets
[a]Migration in the direction of a chemotactic agent
[b]Random migration

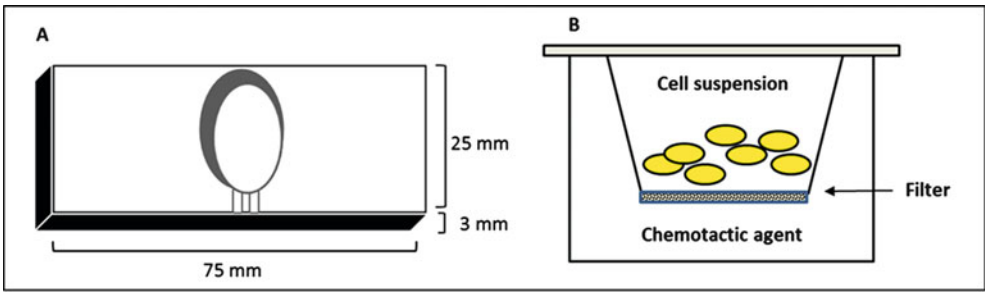

Fig. 3 (**A**) Diagram of the stainless steel slide. (**B**) Boyden chemotaxis chamber

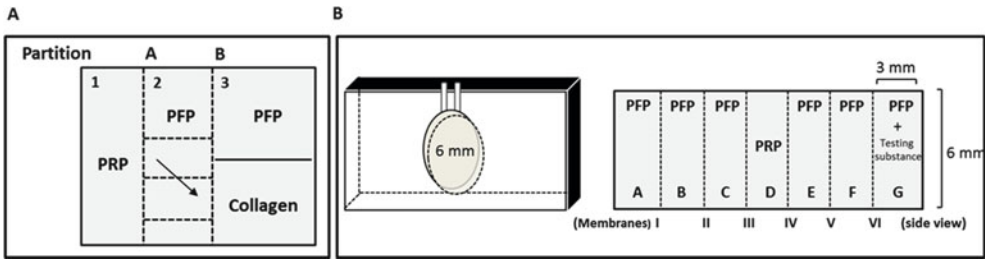

Fig. 4 (**A**) Diagram of the micromaze. PRP is placed in compartment 1, PFP in compartments 2 and 3 and collagen suspended in PFP in compartment 4. (**B**) Seven-compartment chamber. (*sx*) A basic unit of the seven-compartment chamber. (*dx*) The linearly connected 7-compartment chamber showing contents in each compartment

Valone instead studied platelet migration by adapting the Boyden chamber initially developed for the study of leukocyte chemotaxis (Boyden 1962) (Fig. 3b). This is a perspex chamber composed of two compartments separated by a filter membrane 100 μm thick with pores of 8 μm size. A platelet suspension in standard buffer (0.005 M KH_2PO_4, 0.005 M Na_2HPO_4, 0.1 M NaCl, 0.2 g/100 mL glucose and 0.5 g/mL gelatin) (100,000/μL) was placed in the upper compartment and buffer medium in the lower compartment. After 3 h of incubation the filter was removed, washed, fixed in 10 % formalin, stained, cleared and mounted on a glass slide. Platelets migrated into the filter were counted in 10 high-power fields by phase-contrast microscopy at a fixed level (40–70 μm from the top of the filter) (Valone et al. 1974).

Directional migration of platelets towards a chemotactic gradient was also studied with the micromaze method (Lowenhaupt 1978) or the 7-compartment chamber using indium^{111}-oxine-labeled platelets (Lowenhaupt et al. 1982). The micromaze is a chamber formed by four compartments connected by thin slits to permit cell passage between compartments; collagen was placed in compartment 4 (Fig. 4a). Platelet movement was visualized by an inverted phase-contrast microscope by following the leading edge of the platelet mass or by viewing the passage of individual platelets between compartments through the slits: time-lapse image sequences were taken at fixed intervals (Lowenhaupt 1978). The 7-compartment chamber consists of seven identical compartments linearly connected and separated by Nucleopore or Millipore filters of different pore size (III and IV 3 μm, II and V 1 μm, I and VI 0.45 μm); collagen was placed in compartment G (Fig. 4b). Gel-filtered platelets were labeled with ^{111}In-oxine, resuspended in autologous platelet-free plasma (PFP) and filtered through two sterile nylon mesh filters to remove aggregates. Platelet chemotaxis was expressed as a ratio of the radioactive counts of the two-end compartments (Lowenhaupt et al. 1982).

More recently platelet chemotaxis has been studied using several further modifications of the original Boyden chamber, namely:

1. The NeuroProbe 96-well ChemoTx microplate, with the upper and lower compartments separated by a 2 μm pores filter. After 2 h of incubation, platelets migrated into the lower compartment were counted by light microscopy (Czapiga et al. 2005).
2. The Nucleopore single wells, with the upper and lower compartments separated by a 3 μm pore size filter, loaded with murine or human PRP. After 1.5 or 3 h incubation, respectively, filters were stained and platelets were counted at various depths below the filter surface (0–100 μm for murine and 40–70 μm for human platelets) (Pitchford et al. 2008).
3. The transwell inserts, with a polyethylene terephthalate (PET) membrane with 0.4 μm pores. Platelets were added in the upper compartment, allowed to migrate

for 8 h and then counted in the lower compartment by flow cytometry (Kraemer et al. 2010).

Horizontal migration of platelets has been studied in a delta T culture dish placed on a microscope with a heated stage and platelet movements were recordedbefore and during the addition of either fMLP or PBS (as a control) every 5 s for 15 min (Czapiga et al. 2005), or in a migration chamber consisting of a fibrinogen-coated slide with a central spot of low-melting agarose containing the chemotactic agent and platelet movement were recorded using a polarization microscope for 3 h (Kraemer et al. 2010).

The assays used to study platelet migration are summarized in Table 1.

Chemotactic Agents

Platelets can detect an extracellular chemotactic gradient and move along this gradient. The first platelet chemotactic agent to be described has been collagen. Various types of collagen (bovine, equine, human from skin or from achilles tendon) elicit platelet chemotaxis, although with different potency. Only native collagen, and not heat-denatured or dinitrofluorobenzene-treated collagen, induces platelet chemotaxis. Interestingly, the structural features of collagen required for platelet aggregation, i.e. the fibrillar structure, are not required for chemotaxis. Collagen-induced platelet chemotaxis does not require a direct contact with platelets, given that migration was still observed when a filter impermeable to the large polymerized collagen molecules was interposed between platelets and the stimulus. Thus, the generation of "chemotaxins", low molecular weight substances produced by the interaction between collagen and plasma, was postulated (Lowenhaupt 1982). Platelets migrated in the direction of collagen for a long distance (3000 times their diameter, i.e. 6 mm) in a very short time (15 min) (Lowenhaupt 1978).

Formyl peptides, cleavage products of bacterial and mitochondrial proteins, induce platelet chemotaxis via binding to formyl peptide receptors (FPR), seven transmembrane receptors coupled to Gαi stored in α-granules and expressed on the platelet surface after activation (Czapiga et al. 2005). Platelet movement towards fMLP at a velocity of 13.07 ± 1.10 μm/min has also been recorded in time-lapse (Czapiga et al. 2005).

Recently, platelet chemotaxis towards a conventional chemokine of the CXC family, CXCL12 or stromal cell-derived factor-1α (SDF-1α), has been shown (Kraemer et al. 2010). This chemokine induced platelet migration upon binding to its specific receptor CXCR4 expressed on platelets, given that the CXCR4-receptor antagonist AMD3100 inhibits it. Platelets also trans-migrate through an IL-1β-activated layer of human umbilical vein endothelial cells (HUVEC) in the direction of SDF-1α (Kraemer et al. 2010). In time-lapse studies platelets accumulated around the source of SDF-1α after 3 h, with a speed of migration variable depending on the number of focal adhesion contacts. In the early stages of migration (fast migration: 200 μm/3 h) platelets have only few focal adhesion contacts, while their number increases as migration speed slows down (Kraemer et al. 2010).

Molecular Mechanisms Regulating Platelet Migration

Platelet migration is an active, energy-consuming process that requires viable and metabolically intact platelets. Infact, fixation with formalin or pre-treatment with iodoacetic acid (IAA) and sodium fluoride (NaF), which interfere with the glycolytic pathway, with 2,4-dinitrophenol (DNP), which uncouples oxidative phosphorylation, and with 6-aminonicotinamide, which suppresses the hexose monophosphate shunt, blocks platelet migration (Valone et al. 1974; Lowenhaupt et al. 1977). As expected cytochalasin B, which disrupts actin filaments, inhibited migration, while colchicine, which interferes with the polymerization of microtubules, did not (Lowenhaupt et al. 1977).

Platelet migration triggered by SDF-1α is mediated by PI3K, given that the PI3K inhibitors wortmannin and LY294002 significantly inhibited it. PI3K phosphorylates Wiskott-Aldrich syndrome protein (WASP) that induces the rearrangement of the actin cytoskeleton (Kraemer et al. 2010). Downstream signaling linking PI3K to platelet migration involves the serum- and glucocorticoid-inducible kinase 1 (SGK1), known to be involved in endothelial cell and monocyte/macrophage migration (Borst et al. 2015; Zarrinpashneh et al. 2013). The importance of SGK1 in platelet migration seems to be connected to its ability to regulate the actin cytoskeletal architecture in fact WASP and vinculin, two proteins interacting with actin, are SGK1-sensitive. WASP activates the Arp 2/3 complex that binds actin, thus inducing its polymerization, and vinculin is an actin-binding protein that, when phosphorylated, stabilizes the focal adhesions (Kraemer et al. 2010; Schmidt et al. 2012). SGK1-deficient platelets show impaired migration, reduced WASP activation and enhanced vinculin phosphorylation (Schmidt et al. 2012).

Platelet migration is dependent on the increase of intracellular Ca^{2+} via the Ca^{2+} channel Orai1, the pore forming unit of the store-operated Ca^{2+} entry (SOCE) channel, and on K^+ efflux via the Ca^{2+}-activated K^+ channel SK4 (Schmidt et al. 2011).

Platelets adhering to a fibrinogen-coated surface and then exposed to high shear conditions ($1500\ s^{-1}$) undergo

polarization, cytoskeletal reorganization with increased WASP phosphorylation and redistribution of intracellular focal adhesion kinases (FAK) to areas of dynamic focal adhesions, and migration in the direction of flow at a speed of approximately 10 μm/h (Kraemer et al. 2011).

Platelet Migration in Disease

Platelet migration has also been studied in disease conditions and/or in response to various pathologic stimuli. Duquesnoy in 1975 described a platelet migration inhibition (PMI) assay to detect antibodies in serum directed against the human leukocyte antigen (HLA) and the platelet-specific antigen Pl-A1 (Duquesnoy et al. 1975), or alloantibodies against platelets in platelet-transfused patients (Levine and Brubaker 1983). This assay was a modification of the capillary tube chemotaxis chamber used by Lowenhaupt et al. (1973) and it tested the capacity of antibodies to inhibit platelet migration by mixing control PRP with patient's serum. The sensitivity of the PMI test was reported to be comparable or even greater to that of several other methods used for the detection of platelet antibodies, such as the platelet lysis assay, complement fixation, platelet aggregometry and platelet factor-3 release (Duquesnoy et al. 1975; Levine and Brubaker 1983).

Platelets from allergic asthmatic subjects, but not from healthy donors, concentration-dependently migrated in vitro in response to the specific sensitizing allergen and in response to a monoclonal anti-human IgE antibody. In asthmatic subjects allergen-specific IgEs, produced upon previous contact with the allergen and bound to the platelet high-affinity receptor for IgE, FcεRI, bind the allergen inducing the cross-linking of contiguous receptors thus triggering platelet chemotaxis (Pitchford et al. 2008). The same phenomenon is triggered by an anti-IgE antibody that, binding to contiguous Fc portions of FcεRI-bound IgEs, induces the cross-linking of the receptors. The crosslinking of IgE receptors on platelets was previously shown to trigger other platelet functional activities, such as cytotoxicity (Polack et al. 1991), oxygen radical formation (Vargas et al. 1999) and release of chemokines (Hasegawa et al. 2001; Klouche et al. 1997). Migration of platelets from ovalbumin (OVA)-immunized mice in response to the sensitizing allergen was also demonstrated (Pitchford et al. 2008).

Platelet Chemotaxis: Studies in Animal Models

Studies in animal models confirm the ability of platelets to migrate into inflammed tissues. Extravascular accumulation of platelets in bronchial tissue and in lungs, associated with bronchospasm, has been observed by electron microscopy or

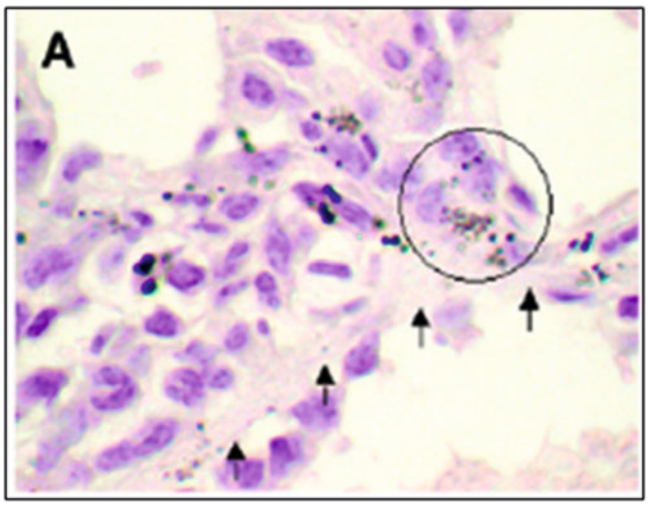

Fig. 5 Individual platelets (*arrows*) migrated in lung parenchyma after mouse allergen challenge. Reprinted with permission of the American Thoracic Society. Copyright (c) 2016 American Thoracic Society. Pitchford SC et al., 2008, Allergen induces the migration of platelets to lung tissue in allergic asthma, Am Respir Crit Care Med 177; 604–612. The American Journal of Respiratory and Critical Care Medicine is an official journal of the American Thoracic Society

by the accumulation of ^{111}In-oxine-labelled platelets in guinea pigs and baboons after intravenous challenge with platelet-activating factor (PAF) and other platelet agonists, like ADP or collagen. The penetration of platelets in tissue was not the consequence of blood extravasation, as no other blood cells were concomitantly found (Arnoux et al. 1988; Lellouch-Tubiana et al. 1985; Page et al. 1984; Robertson and Page 1987). The presence of platelets in skin accompanied by neutrophils was also described after the intradermal injection of PAF in rats (Pirotzky et al. 1984).

Platelets have also been detected in bronchoalveolar lavage (BAL) fluid of mice with chronic allergic airway inflammation (Pitchford et al. 2004) and from rabbits with experimental asthma, following allergen-challenge (Coyle et al. 1990).

Platelets from OVA-immunized mice were observed to migrate out of blood vessels after allergen inhalation and to localize in lung parenchima, directly underneath the airways (Fig. 5). Platelet influx in tissue preceded leukocytes and was largely independent of the latter (Pitchford et al. 2003, 2005). Platelet migration into inflammed lung was shown to be mediated by the binding of allergen to FcεRIγ-bound allergen-specific IgEs on the platelet surface (Pitchford et al. 2008), a phenomenon previously described for other inflammatory cells such as eosinophils, basophils and mast cells (Ishizuka et al. 2001; Orida et al. 1983; Svensson et al. 2004).

Transendothelial migration of platelets into the skin of guinea pigs induced by the subcutaneous injection of fMLP was demonstrated by serial electron-microscopy of thin tissue sections (Feng et al. 1998b). In that study it was shown

that platelets crossed endothelial cells not at the level of interendothelial cell junctions, which remained closed. However, platelets have been demonstrated to extend pseudopods suggesting active diapedesis. Moreover, single platelets enclosed within endothelial cytoplasmic vacuoles, generally located close to interendothelial cell junctions, were observed and the platelet-containing vacuoles were observed to open to the abluminal surface whereupon platelets were discharged into the underlying basal lamina. Following transmigration across the basal lamina, platelets were found free in dermal connective tissue, together with neutrophils and other white cells. Interestingly, migrating platelets did not display the ultrastructural features of a release reaction, suggesting that conventional platelet activation is not required for platelet migration (Feng et al. 1998b).

The mechanism of transmigration observed in this model, i.e. that platelets cross undamaged endothelium by a process similar to pinocytosis, has been previously described for neutrophils (Feng et al. 1998a). This mechanism does not necessarily apply to all stimuli-inducing diapedesis and active migration through interendothelial cell junctions may also take place (Laitinen 1993; Marchesi 1966).

Platelet translocation into the Disse spaces of the liver and their active penetration into hepatocytes have been reported by immunostaining for 5-hydroxytryptamine (5-HT), a sensitive method to detect platelets in tissue as platelets contain large amounts of 5-HT, and by electron microscopy (Nakamura et al. 1998). Platelets in the Disse spaces of lipopolysaccharide (LPS)-treated mice were in contact with Kupffer cells (hepatic macrophages) (Nakamura et al. 1998; Yamaguchi et al. 2006). This process seems to involve biochemical pathways different from those involved in aggregation, given that anti-platelet agents, including aspirin, did not prevent hepatic platelet accumulation (Nakamura et al. 1998).

In a murine model of corneal abrasion, diapedesis of platelets out of vessels was demonstrated with accumulation of platelets in the limbus where they actively contribute to corneal nerve regeneration. The accumulation of platelets was mediated by P-selectin (Li et al. 2011), an adhesion molecule that also plays a role in the accumulation of platelets in glomeruli in a murine model of glomerulonephritis (Zachem et al. 1997). Activated platelets, alone or together with neutrophils, were found by immunofluorescence within glomeruli of rats with nephritis induced by the selective perfusion of the renal artery with the lectin concanavalin A (Zachem et al. 1997).

In a model of ligation of intestinal arteries in mice, green fluorescent protein (GFP)-labeled platelets were observed in areas of post-ischemic inflamed tissue, where they could function as pilot cells that guide the invasion of other inflammatory cells. This mechanism was mediated by SDF-1α involving signalling through PI3K and activation of SGK-1 (Kraemer et al. 2010). Furthermore, SGK-$1^{-/-}$ mice showed decreased platelet transmigration into the ischemic intestinal vascular wall (Schmidt et al. 2012).

Platelet Chemotaxis: Studies in Humans

Despite the difficulty in detecting platelets in tissue using histology with conventional staining techniques, due to the small dimensions and the lack of a nucleus, observations using electron microscopy and/or immunological staining confirm the ability of platelets to transmigrate into tissues in humans with inflammatory conditions.

Platelets were found in BAL of patients with allergic asthma following allergen challenge without the concomitant presence of erythrocytes, confirming active diapedesis and not passive transfer due to blood extravasation, with some degranulated platelets and free granules in the lavage (Metzger et al. 1985, 1987). Platelet aggregates have also been observed in the lamina propria of the microvasculature of lungs of asthmatic subjects by transmission electron microscopy during late-onset airways obstruction following allergen provocation, in apposition to areas of bronchial smooth muscle, underneath the epithelium, and in areas of eosinophil infiltration (Beasley et al. 1989).

Extravascular platelets, colocalized with leukocytes, have been detected by immunofluorescence in surgically excised nasal polyps from patients with aspirin-exacerbated respiratory disease (AERD), a chronic inflammatory disorder characterized by nasal polyposis and asthma triggered by the ingestion of aspirin (Laidlaw and Boyce 2012).

Platelets were also identified by immunohistochemistry in brain tissue sections from patients with multiple sclerosis with active demyelinating plaques and by confocal immunofluorescent microscopy in a chronic active type 1 lesion (active inflammation and demyelination) (Langer et al. 2012).

Electron microscopy of the synovium of patients with rheumatoid arthritis (RA) showed platelet thrombi obliterating the lumen of vessels and platelets were observed in the vicinity of gaps between endothelial cells of the joint vasculature (Schumacher 1975). Positive staining for $\alpha_{IIb}\beta_3$ outside the vasculature was detected using immunohistochemistry of the synovium from patients with RA, representing either platelets or platelet-derived microparticles (PMPs) (Konttinen et al. 1989; Palmer et al. 1986). Moreover, platelets, platelet aggregates and platelet-leukocytes complexes have been shown in the synovial fluid of patients with RA suggesting active migration into extravascular sites (Endresen 1981; Endresen and Forre 1992; Farr et al. 1984; Ginsberg et al. 1978; Yaron and Djaldetti 1978).

Platelet Contribution to the Chemotaxis of Other Cells

Platelets influence the migration of other cells by releasing soluble chemotactic mediators, by liberating PMPs, or by direct contact with the involved cells (Fig. 6).

Platelet lysates enhanced cell migration of several hepatocellular carcinoma cell lines (Carr et al. 2014) and fibroblasts (Carducci et al. 2016; Senior et al. 1983), and adherent activated platelets stimulated the migration of murine embryonic endothelial progenitor cells (EPC) (Langer et al. 2006).

Platelets contain and release upon activation several soluble mediators influencing cell migration (Table 2). Among them chemokines, which represent a significant fraction of the platelet α-granules content, are released upon platelet activation and mediate the recruitment of several cells to sites of inflammation, including leukocytes, cancer cells and hematopoietic cells, thus favouring neointima formation and atherosclerosis, vessel repair and regeneration after vascular injury (Gleissner et al. 2008). CXCL4/platelet factor 4 (CXCL4/PF4), the first member of the chemokine family discovered in platelets and the most abundant platelet chemokine (Deuel et al. 1977), and CXCL7/neutrophil-activating peptide-2 (CXCL7/NAP-2) purified from supernatants of thrombin-stimulated platelets induce neutrophils to undergo firm adhesion on an endothelial cells monolayer in a concentration-dependent manner. CXCL7 also stimulates neutrophil transendothelial migration (Petersen et al. 1999; Schenk et al. 2002). Furthermore, CXCL7 and CXCL5/epithelial neutrophil-activating protein 78 (ENA-78), secreted by activated platelets upon contact with tumor cells, induce

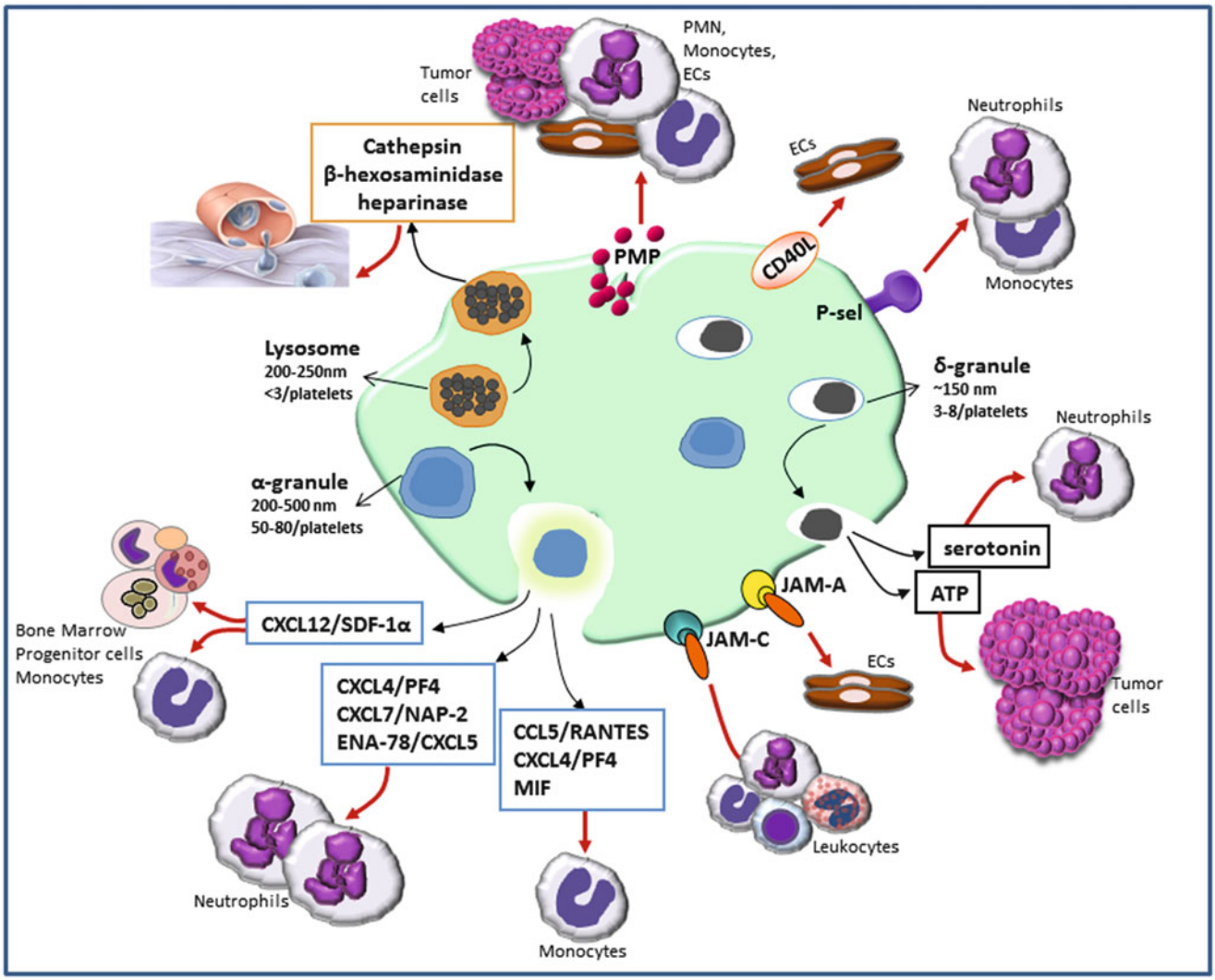

Fig. 6 Platelet contribution to the chemotaxis of other cells. Platelets play an active role in the induction of chemotaxis of other cells by releasing, upon activation, soluble mediators contained in their granules (α, δ and lysosomes), by liberating PMPs, and by expressing surface receptors favouring cell–cell interactions

Table 2 Soluble platelet-derived inflammatory mediators and platelet surface proteins that modulate cell migration

Soluble platelet-derived mediators	Responding cells
α-granules	
CXCL4/PF4	Neutrophil firm adhesion on the endothelium
CXCL7/NAP-2	Neutrophil firm adhesion on the endothelium and trans-migration
	Formation of the early metastatic niche
CXCL12/SDF-1α	Adhesion and migration of bone marrow progenitor cells
	Monocyte adhesion and chemotaxis
CXCL5/ENA-78	Neutrophil migration
	Formation of the early metastatic niche
CCL5/RANTES	Monocyte adhesion on the endothelium and recruitment
MIF	Monocyte arrest on the endothelium and chemotaxis
Dense granules	
Serotonin	Neutrophil and T-cell recruitment
ATP	Tumor cell transendothelial migration and metastasis
Lysosomes	
Cathepsin, heparinase, β-hexosoaminidase	Cell diapedesis by remodeling the inflammed tissue
PMPs	Neutrophil and monocyte adhesion to the endothelium
	Chemotaxis and invasion of breast and lung cancer cells
	Chemotaxis of hematopoietic stem/progenitor ($CD34^+$) and various myeloid and lymphoid cells
Platelet surface proteins	
CD40L/CD154	Endothelial cell activation
CD40	T cell recruitment
P-selectin	Monocyte and neutrophil rolling and adhesion to the endothelium
JAM-A	Platelet adhesion to the endothelium
JAM-C	Firm adhesion of leukocytes on adherent platelets

granulocyte migration and guide the formation of the early metastatic niche (Labelle et al. 2014). Activated platelets are a major source of CXCL12/SDF-1α (Chatterjee and Gawaz 2013). Platelet-derived CXCL12 supports adhesion of CD34+ human progenitor cells (PCs) under static conditions and facilitates the rolling and firm adhesion of CD34+ cells onto platelets adhered to a layer of human aortic endothelial cells (HAEC) under high shear rate in vitro and in vivo (Stellos et al. 2008). Platelet-derived CXCL12 also enhances the adhesion and migration of bone marrow progenitor cells to sites of vascular injury thereby promoting repair (Massberg et al. 2006). Furthermore, CXCL12 released by activated platelets induces monocyte chemotaxis by acting on CXCR4 and monocyte adhesion under static and dynamic arterial flow conditions by acting on CXCR7 (Chatterjee et al. 2015). CCL5/regulated on activation normal T cell expressed and secreted (CCL5/RANTES) secreted by activated platelets and immobilized on the inflamed/activated endothelium of atherosclerotic arteries induces adhesion of monocytes (Mause et al. 2005; Schober et al. 2002; von Hundelshausen et al. 2001). Platelets under shear flow deposit CXCL4 and CCL5 on atherosclerotic or IL-1β-activated HAEC, enhancing the recruitment of monocytes to the endothelium (Baltus et al. 2005; Huo et al. 2003). Moreover, platelet-derived macrophage migration inhibitory factor (MIF) stimulates monocyte arrest on endothelium and chemotaxis (Wirtz et al. 2015).

Platelet-derived IL-1β induces the secretion of CCL2/monocyte chemottractant protein-1 (MCP-1) and increases the expression of intracellular adhesion molecule-1 (ICAM-1) by endothelial cells, promoting the adhesion of monocytes to the endothelium and their chemotaxis; in fact MCP-1 is a potent chemotactic factor for monocytes (Gawaz et al. 2000). Platelet-derived IL-1 induces the release of CXCL1 and CXCL8 from endothelium, which in turn induces neutrophil recruitment (Page and Pitchford 2013; Kaplanski et al. 1993; Thornton et al. 2010).

Platelet dense-granules contain serotonin, a vasoactive inflammatory mediator that can induce vascular permeability and promotes the recruitment of neutrophils into lung and peritoneum, after intraperitoneal and intratracheal LPS administration, and in aseptic skin wounds (Duerschmied et al. 2013), and the recruitment of T cells into the liver during viral hepatitis-induced hepatic injury (Lang et al. 2008). Platelet-derived ATP promotes tumor cell transendothelial migration and metastasis via stimulation of P2Y2 receptors (Schumacher et al. 2013).

Platelet release lysosomal enzymes, such as cathepsin, β-hexosoaminidase and heparinase, in vivo in humans at a localized site of vessel wall damage (Ciferri et al. 2000; Vlodavsky et al. 1992), and these may participate in cell diapedesis due to their tissue-degrading activity and by remodelling the inflammed tissues, a role already demonstrated to be involved in the migration of fibroblasts,

cancer and endothelial cells (Mohamed and Sloane 2006; Palka et al. 1997; Schraufstatter et al. 2003)

PMPs play an important role in tissue recruitment of inflammatory cells by the interaction between P-selectin expressed on their surface and P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils (Forlow et al. 2000) and simultaneously by their adhesion to the subendothelial matrix through integrin $\alpha_{IIb}\beta_3$ (Merten et al. 1999). PMPs stimulate monocyte adhesion also by inducing endothelial cells (ECs) to express ICAM-1 and by delivering chemokines, such as RANTES, to the endothelium (Barry et al. 1998; Mause et al. 2005). In addition, PMPs transport cytokines (e.g. IL-1β) that stimulate polymorphonuclear cells (PMNs) adhesion to ECs and miRNAs that may modify the phenotype of endothelial cells (Gidlof et al. 2013) and macrophages (Laffon et al. 2016). PMPs enhance the chemotaxis of invasive breast and lung cancer cells and stimulate their invasion across Matrigel by inducing MMPs production. Furthermore, PMPs act as chemotactic agent for hematopoietic stem/progenitor ($CD34^+$) cells as well as for various myeloid and lymphoid cells (Baj-Krzyworzeka et al. 2002; Janowska-Wieczorek et al. 2005, 2006).

Platelets express upon activation a number of surface proteins involved in heterotypic interactions with endothelial cells and leukocytes, mediating the rolling and adhesion of leukocytes to the endothelium and the subsequent transmigration into inflammed tissue (Gawaz et al. 2005; Weyrich and Zimmerman 2004). The formation of platelet-neutrophil and platelet-monocyte complexes, the subsequent neutrophil and monocyte adhesion to the endothelium and recruitment into the inflammed tissue is dependent on platelet P-selectin (Page and Pitchford 2013), mediated by its binding to the high affinity counter ligand PSGL-1 (Hamburger and McEver 1990; Moore et al. 1995; Kuijper et al. 1998). High-resolution videomicroscopy has revealed the existence of membrane tethers involving P-selectin/PSGL-1 bonds that regulate neutrophil rolling on platelets (Schmidtke and Diamond 2000). The importance of platelet P-selectin/PSGL-1 axis has been determined by the use of P-selectin-deficient mice, by the blockade of P-selectin or with PSGL-1 antibodies (Abdulla et al. 2012; Diacovo et al. 1996; Mayadas et al. 1993; Pitchford et al. 2005).

Platelet CD40 ligand (CD40L/CD154) binds CD40 on endothelial cells and enhances the release of IL-8 and MCP-1, the expression of E-selectin (CD62-E), Vascular Cell Adhesion Protein-1 (VCAM-1) and ICAM-1, and the release of matrix metalloproteinases (e.g., MMP-1, -2, -3, and -9). Furthermore CD40L-positive T cells activate platelets through a CD40-dependent pathway resulting in CCL5 release and T cell recruitment (Danese et al. 2004; Henn et al. 1998; Giannini et al. 2011).

Platelet Junctional Adhesion Molecule (JAM-A) can support homophilic interactions with endothelial-cell JAM-A, mediating platelet adhesion to the endothelium (Babinska et al. 2002), thus facilitating the deposition on endothelium of platelet CCL5/RANTES (Zernecke et al. 2006). Platelet JAM-C functions as a counter-receptor for the β2-integrin Mac-1 on neutrophils mediating firm adhesion of leukocytes to adherent platelets (Santoso et al. 2002).

Conclusions

Among the functions that characterize platelets as inflammatory cells, one which is little considered but is probably crucial is the ability to migrate into tissue in the direction of a chemotactic stimulus. This allows platelets to actively participate in the tissue inflammatory process by releasing stored or newly synthesized mediators acting both on other platelets and/or on other cell types. Furthermore, platelets influence and sometimes are essential for the migration of other inflammatory cells, including leukocytes and cancer cells.

This "non-classical" platelet activity is an example of the existence of a dichotomy in platelet function, i.e. the ability of platelets to display an inflammatory or a haemostatic/thrombotic response depending on the stimulus and on the environment, recently elegantly demonstrated investigating the role of different purinergic receptor subtypes on platelets (Amison et al. 2015).

Further investigation into the mechanisms regulating platelet migration, and in general the characterization of the mechanisms regulating this dichotomy of platelet function, may be crucial for the discovery of new therapeutic approaches to inflammatory diseases by the development of drugs able to interfere with the inflammatory but not with the haemostatic function of platelets.

Take Home Messages

- Platelets possess several characteristics that allow them to migrate: the expression of receptors for chemokines and for other chemotactic agents, the presence of all the signaling pathways responsible for the transduction of the extracellular chemotactic signal to the motile apparatus, a dynamic cytoskeleton and the relase of several enzymes (MMPs, cathepsins, β-hexosoaminidase, heparinase) responsible for ECM degradation.
- The ability of platelets to migrate in vitro, both randomly and in the direction of a chemotactic stimulus, such as collagen, fMLP, SDF-1α, IgE and allergens, has been confirmed using different assays.
- The penetration of platelets in inflammed tissues has been described in several animal models and in human disease conditions.

(continued)

- Platelets can induce the migration of other cell types by several mechanisms, including the shedding of PMPs, release of granular materials (chemokines, cytokines, growth factors, ATP, enzymes), and the expression of surface receptors involved in platelet heterotypic interactions with leukocytes, endothelial and cancer cells.

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Platelet Secretion

Robert Flaumenhaft

Abstract

Granule secretion is a critical platelet function, essential in both physiological and pathophysiological activities of platelets. Granules constitute more than 10 % of total platelet volume and account for nearly a third of total platelet membrane. The classic platelet granule types include dense granules, α-granules, and lysosomes. Dense granules contain high concentrations of bioactive amines, cations, polyphosphates, and nucleotides. α-Granules contain hundreds of different proteins, including coagulation factors, growth factors, proteases, and transmembrane receptors. Lysosomes represent a minor granule population that contain enzymes involved in the degradation of proteins, carbohydrate, and lipids. With this diversity of contents, platelet granules participate in many functions, including hemostasis and thrombosis, inflammation, atherosclerosis, antimicrobial host defense, malignancy, angiogenesis, and wound healing. The secretion of platelet granules is tightly controlled so as to permit rapid release of contents in response to injury while preventing inadvertent release of potentially deleterious contents. This chapter will review established and recent studies describing platelet granule content and function, the mechanisms of granule secretion from platelets, and human platelet granule deficiency syndromes.

Platelet Granules

The application of electron microscopy in the 1960s enabled our current classification of platelet granules into dense granules (aka, δ-granules or dense core granules), α-granules, and lysosomes (Tranzer et al. 1966). Dense granules were readily detected on the basis of their intrinsic electron density. α-Granules were identified as an atypical granule, reminiscent of but distinct from lysosomes (Siegel and Luscher 1967). In fact, it was not until cytochemistry was coupled to electron microscopy that lysosomes and α-granules were convincingly distinguished (Bentfeld-Barker and Bainton 1982). While the existence of additional granule types and subpopulations of granules within the platelet remains an area of active investigation, dense granules, α-granules, and lysosomes remain the three universally recognized platelet granules.

- Dense granules and α-granules are lysosome-related organelles found only in platelets.
- Both dense granules and α-granules are required for normal hemostasis and thrombosis.
- Dense granules contain primarily bioactive small molecules and cations.
- α-Granules contain a matrix of tightly packed and varied proteins that participate in a broad range of physiological activities.
- Lysosomes contain enzymes for degrading proteins, carbohydrates, and lipids.

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Dense Granules

Although platelet dense granules are unique to platelets, they belong to a class of granules termed lysosome-related organelles that are found in other secretory cells such as melanosomes and chromaffin cells (Raposo et al. 2007). Platelets contain an average of 3–8 dense granules (Gerrard et al. 1977; White and Gerrard 1978). These granules are approximately 150 nm in diameter and are electron dense. Dense granules are acidic, with a luminal pH of ~5.4 (Dean et al. 1984). They express a vesicular H^+-ATPase proton pump that maintains the pH gradient across granule (Fig. 1) and generates a luminal positive electrochemical gradient ($\Delta\Psi$) of 30–40 mV (Carty et al. 1981). Newer studies implicate two-pore channel 2 (TPC2) as an important regulator of dense granule luminal pH and Ca^{2+} flux from dense granules (Fig. 1) (Ambrosio et al. 2015). In addition to H^+, platelet dense granules concentrate high levels of calcium (2.2 M), magnesium, and potassium (Fukami et al. 1984; Ruiz et al. 2004), which can be free or complexed to anions.

The electron gradient across the dense granule membrane powers transporters that are responsible for the substantial concentration of cytosolic nucleotides and bioactive amines observed in the granules. Anions in platelet dense granules include adenosine nucleotides, which are concentrated at ~653 mM ADP and ~436 mM ATP (Holmsen and Weiss 1979; Fukami et al. 1984; Holmsen and Dangelmaier 1989; Lages and Weiss 1999). VNUT is a 12 transmembrane domain transporter (Van Liefferinge et al. 2013) that was first predicted to reside on platelet dense granules based on their similarity to the large dense core vesicles of chromaffin cells (Fitch-Tewfik and Flaumenhaft 2013) and subsequently found to reside on platelet dense granules (Hiasa et al. 2014). Glyoxylate, an inhibitor of VNUT, blocks ATP uptake into megakaryocyte-like cells (Hiasa et al. 2014). MRP4 is a second nucleotide transporter found on dense granule membranes. It was originally thought to function in loading adenosine nucleotides into dense granules but was more recently shown to serve an essential role in cAMP homeostasis (Decouture et al. 2015). Polyphosphate and pyrophosphates are present in dense granules in millimolar

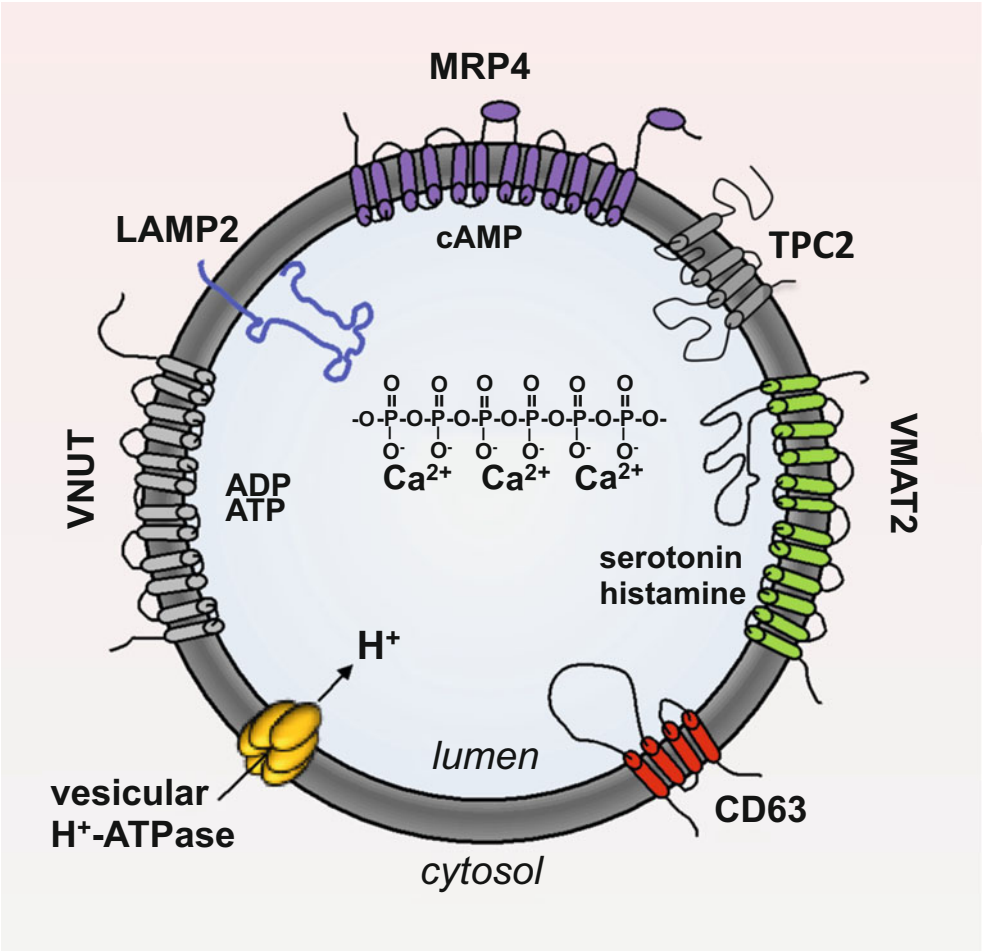

Fig. 1 Platelet dense granule. Platelet dense granules are the storage unit for the ionic species (Ca^{2+}, polyphosphate), adenosine nucleotides (ADP and ATP), and bioactive amines including serotonin and histamine. A vesicular H^+-ATPase proton pump and two-pore channel 2 (TPC2) maintains a low intraluminal pH and a transmembrane electrochemical gradient. TPC2 also functions in Ca^{2+} from dense granules. VNUT uses the electrochemical gradient to facilitate concentration of ADP and ATP in dense granules. MRP4 participates in cAMP homeostasis. VMAT2 is responsible for the transfer of serotonin from cytosol into dense granules. Membrane proteins include CD63 and LAMP-2, which is highly glycosylated and protects the membrane from the acid environment (Adapted from Fitch-Tewfik and Flaumenhaft 2013)

concentrations (in terms of Pi residues) and are typically ~60–100 phosphate units long (Muller et al. 2009; Morrissey et al. 2012), although their length can vary substantially. Mice lacking inositol hexakisphosphate kinase 1 have reduced levels of polyphosphate (Ghosh et al. 2013), implicating inositol hexakisphosphate kinase 1 in their synthesis. The mechanisms by which polyphosphophosphates are concentrated in dense granules are poorly understood. Of note, cytosol contains high concentrations of ATP and relatively little ADP and both ATP and ADP accumulate at high concentrations in dense granules. This observation has led to the theory that VNUT transports primarily ATP into dense granules and hydrolysis of ATP provides for both intraluminal ADP and phosphate substrates for polyphosphate synthesis (Hiasa et al. 2014).

Bioactive amines represent another important class of dense granules contents. Serotonin is present in platelet dense granules at a concentration of ~0.5 M (Ge et al. 2008, 2009). Vesicular monoamine transporter 2 (VMAT2) mediates uptake of serotonin into platelet dense granules (Fig. 1) (Leitner et al. 1999). During the process of serotonin concentration in dense granules, serotonin is first pumped into the cytosol from plasma by a serotonin transporter (SERT). The electrochemical proton gradient across the dense granule membrane then drives VMAT2-mediated uptake of serotonin from cytosol into platelet granules. Histamine uptake into dense granules is also mediated by VMAT-2 (Fukami et al. 1984; Tharmapathy et al. 2000). VMAT2 is targeted to dense granules by virtue of signals encoded in the C-terminal domain of the protein such as a C-terminal dileucine motif (Ambrosio et al. 2012). The C-terminus of VMAT2 also contains an acidic motif including two serines that is required for retention in granule membranes (Waites et al. 2001).

Platelet dense granules contain several other membrane proteins in addition to transporters. Both tetraspanin CD63 (granulophysin) and lysosomal-associated membrane protein-2 (LAMP-2, CD107b) are expressed on dense granule membranes (Fig. 1). CD63 is a member of the tetraspanin family that contains four membrane-spanning domains and luminal/extracellular domains that are extensively glycosylated. CD63 functions in membrane trafficking and is expressed on the platelet surface following activation where it associates with $\alpha_{IIb}\beta_3$ (Israels et al. 2001; van der Zee et al. 2006). Like other tetraspanins, platelet CD63 participates in lipid raft formation (Israels and McMillan-Ward 2007). LAMP-2 contains a single transmembrane domain and is also extensively glycosylated. LAMP-2- functions to maintain the integrity of lysosomal, and presumably dense granule, membranes during vesicle trafficking. Of note, both CD63 and LAMP-2 are also found on platelets lysosomes as well as dense granules. Adhesive receptors such as GPIb and $\alpha_{IIb}\beta_3$ have also been reported to reside on platelet dense granule membranes (Youssefian et al. 1997). However, the contribution of this pool of adhesion receptors to platelet function is unknown.

α-Granules

Platelets contain approximately 50–80 granules in a single platelet. These granules have a diameter of 200–500 nm and surface area of ~14 μm^2/platelet (Harrison and Cramer 1993; Blair and Flaumenhaft 2009. The classic α-granule was described as containing distinct regions based on TEM demonstrating a peripheral membrane, an electron dense nucleoid (containing chemokines and proteoglycans), an electron lucent zone (containing von Willebrand factor), and an intermediate zone (containing additional proteins such as fibrinogen). Yet advances in high-resolution imaging technologies have called into question these earlier findings. Electron tomography with three-dimensional reconstruction of the platelet interior (van Nispen tot Pannerden et al. 2010) demonstrates multiple forms of α-granule populations, including subtypes with multiple luminal vesicles, those with crystalline cross-striations, and forms with elongated tubular granules—previously identified as elongated dense granules (White 2010). Furthermore, superresolution fluorescence microscopy performed to evaluate granule cargos has raised the possibility that platelets contain different subpopulations of α-granules (Sehgal and Storrie 2007). These observations raise fundamental questions about the exact identity and definition of α-granules.

Like dense granules, α-granules are considered lysosome-related organelles. They share several features of dense granules including an acidic interior generated by a Ca^{2+}- or Mg^{2+}-sensitive H^+-pumping ATPase responsible for an electrochemical gradient across the α-granule membrane (Grinstein et al. 1982, 1983; Grinstein and Furuya 1983). Platelet α-granules contain high concentrations of cations (14 mM Ca^{2+} and >60 mM Mg^{2+}) (Grinstein et al. 1983). However, while dense granules are known for their high content of small molecules, α-granules are known for their abundant protein content.

The platelet α-granule proteome consists of over 300 hundred proteins including both membrane-bound proteins and soluble proteins that are secreted into the extracellular space (Table 1) (Coppinger et al. 2004; Maynard et al. 2007). The majority of α-granule proteins are synthesized in megakaryocytes and trafficked into α-granules via the regulated secretory pathway. However, proteins such as fibrinogen, IgG, and albumin are taken up by the receptor-mediated endocytosis. The high concentration of protein within α-granules raises the question of how these granules are capable of storing these proteins at such a high concentration and still maintain an osmolarity roughly equivalent to

Table 1 Contents of platelet α-granules

Type	Examples
Adhesion proteins	fibrinogen, von Willebrand factor, thrombospondin
Chemokines	CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), CXCL12 (SDF-1α), CCL2 (MCP-1), CCL3 (MIP-1α), and CCL5 (RANTES)
Coagulants, anticoagulants, and fibrinolytic proteins	factor V, factor IX, factor XIII, antithrombin, protein S, tissue factor pathway inhibitor, plasminogen, plasminogen activator inhibitor 1, α_2-macroglobulin
Growth factors	vascular endothelium growth factor, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, and insulin-like growth factor, transforming growth factor β
Immune mediators	complement C3 precursor, complement C4 precursor, β1H Globulin, factor D, factor H, C1 inhibitor, IgG
Integral membrane proteins	αIIbβ3, GPIbα-IX-V, GPVI, TLT-1, P-selectin
Microbicidal proteins	thymosin-β4, thrombocidins 1 and 2 (from NAP-2)

that of the cytosol. Such concentration may be achieved via aggregation and multimerization of proteins during protein sorting (King and Reed 2002). Proteins that multimerize (such as vWf and multimerin) and bind other cargo proteins may help achieve the requisite degree of aggregation within the α-granule lumen. It has recently been appreciated that α-granule cargo content is not static throughout the life of an individual and changes in α-granule cargos associated with disease states have been described (Maynard et al. 2010; Peterson et al. 2010; Burkhart et al. 2014).

α-Granule proteins participate in a broad array of physiological functions. Experimental evidence exists supporting a role of α-granule proteins in hemostasis and thrombosis, inflammation, angiogenesis, wound repair, and antimicrobial host defense. That α-granules participate in hemostasis and thrombosis was initially supported by the observation that patients with gray platelet syndrome, who lack normal α-granules, have a bleeding diathesis (Gerrard et al. 1980; Levy-Toledano et al. 1981; Swank et al. 1996; Nurden and Nurden 2007; Gunay-Aygun et al. 2010, 2011). *NBEAL2* deficient mice (Deppermann et al. 2013; Kahr et al. 2013) and VPS33B-deficient mice (Bem et al. 2015), both of whose platelets lack α-granules, were shown to have defects in hemostasis and thrombosis. α-Granules include both prothrombotic proteins, such as vWf, fibrinogen, factor V, factor IX, and factor XIII, and anticoagulant proteins, such as antithrombin, protein S, and tissue factor pathway inhibitor. They also contain components of the fibrinolytic system such as plasminogen and plasminogen activator inhibitor. Angiogenic factors in α-granules include vascular endothelial growth factor, platelet-derived growth factor, fibroblast growth factor, and transforming growth factor-β among others. α-Granules also contain antiangiogenic factors such as endostatin and platelet factor 4 (PF4). Since α-granules contain both prothrombotic and antithrombotic as well as proangiogenic and antiangiogenic proteins, the question of how cargos with opposing activities can be organized and released so as to effectively promote physiological processes has been raised. Some investigators believe that these cargos are physically separated into different α-granule subpopulations that respond to distinct platelet agonists (Sehgal and Storrie 2007; Italiano et al. 2008; Italiano and Battinelli 2009). Others postulate that cargos are spatially separated within α-granules and the kinetics of their release differs (Jonnalagadda et al. 2012). Whether these or other mechanisms account for the ability of platelets to coordinate the activities of proteins with opposing function remains to be determined.

A role for platelets in inflammation and innate immunity has become increasingly evident (Von Hundelshausen and Weber 2007; von Hundelshausen et al. 2009; Semple and Freedman 2010; Semple et al. 2011). α-Granule cargo is essential in this capacity. In addition to their role in hemostasis, platelets are immune cells and their α-granules contain many mediators of inflammation. Some of the most abundant α-granule contents are chemokines, which include CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP, β-TG, CTAP-III, NAP-2), CXCL8 (IL-8), and CXCL12 (SDF-1α) (Schenk et al. 2002; Massberg et al. 2006; Koenen et al. 2009). Many of these chemokines contribute to the development of atherosclerosis, in which platelets have been shown to serve an important role (Von Hundelshausen and Weber 2007). Platelets α-granules also contain mediators of the complement cascade such as complement C4 precursor, complement C3, C1 inhibitor, factor D, and factor H (Peerschke et al. 2008). Cargos that are directly microbicidal are also secreted from α-granules, such as thymosin-β4 and thrombocidins 1 and 2, which are derived from NAP-2 (Semple et al. 2011). PF4 is required for platelets to kill *Plasmodium falciparum* during malarial infection (McMorran et al. 2009; Love et al. 2012; Derby 2012). It is worth noting that while a few α-granule cargo proteins are specific for platelets (such as PF4), many others are found in and secreted by other cells types or circulate in plasma. The ability of platelets to act as first responders and secrete these cargos rapidly in response to injury or infection, and perhaps to concentrate these proteins at an affected

site distinguishes α-granule-derived constituents from similar proteins from alternative sources.

α-Granules contain a wide variety of transmembrane proteins that serve diverse functions. P-Selectin (CD62P) is perhaps the best-characterized protein found in the α-granule membrane. Its tissue distribution is limited to platelets and endothelial cells. P-selectin is composed of an N-teminal C-type lectin domain, an epidermal growth factor like motif, a series of complement repeats, a transmembrane domain, and a C-terminal cytoplasmic tail (Koedam et al. 1992; Furie et al. 2001). The lectin domain, EGF motif, and complement repeats reside on the luminal face of the α-granule in resting platelets. Following activation, P-selectin translocates to the extracellular surface, such that its lectin domain can interact with PSGL-1 on immune cells, recruiting neutrophils to sites of inflammation (Hrachovinova et al. 2003). Tetraspanins and integrins also reside on the platelet α-granule surface and translocate to the platelet plasma membrane surface upon platelet activation. These proteins contribute to platelet–platelet, platelet–leukocyte, and platelet–endothelial cell interactions. Most α-granule transmembrane receptors, such as $\alpha_{IIb}\beta_3$ and GPIb, are also present on the plasma membrane of the resting platelet and receptors from α-granules are thought to constitute a reserve pool. Other receptors, such as P-selectin CD109 and fibrocystin L, are sequestered in α-granules in resting platelets and their translocation to the plasma membrane serves as a sensitive indicator of platelet activation.

Lysosomes

Although platelets are replete with lysosome-related organelles (dense granules and α-granules), they contain few bona fide lysosomes. A platelet typically contains 0–3 lysosomes, which are 200–250 nm in diameter. These organelles are identified by cytochemical stains such as acid phosphatase or arylsulfatase (Bentfeld-Barker and Bainton 1982). Little is known about lysosome formation during megakaryopoiesis. However, platelet-specific knockout of PIKfyve results in excessive storage of lysosomal enzymes, implicating this phosphatidylinositol kinase in platelet lysosome formation (Min et al. 2014). Platelet lysosomes enzymes are involved in degradation of proteins, carbohydrates, and lipids (Table 2). The most abundant class is the acid hydrolases. Lysosome contents are released during platelet activation in vivo following vascular injury (Holmsen and Day 1968; Ciferri et al. 2000). Lysosomal membranes contain LAMP-1, LAMP-2, and CD63 (Bentfeld-Barker and Bainton 1982; Ciferri et al. 2000), which are highly glycosylated. These proteins decorate the luminal lysosome surface and serve to protect the lysosomal membrane. Some have suggested that enzymes released by lysosomes may serve a role in digestion and resolution of thrombi (Rendu and Brohard-Bohn 2001). However, the role of platelet lysosomes in hemostasis and thrombosis is not known.

Table 2 Contents of platelet lysosomes

Type	Examples
Carbohydrate-degrading enzymes	arabinofuranosidase, fucosidase, galactosidase, glucosidase, glucuronidase, hexosaminidase, mannosidase
Phosphate ester cleaving	acid phosphatase
Protein-degrading enzymes	carboxypeptidase, cathepsins, collagenase, elastase, proline carboxypeptidase

Mechanisms of Platelet Granule Secretion

Platelet granule secretion occurs when membranes of granules fuse with membranes of surface-connected structures, including plasma membrane and the open canalicular system (OCS). The fusion of two lipid membranes in an aqueous environment requires sufficient energy to overcome electrostatic repulsive and hydration forces between the two membranes. Platelet membrane fusion must be tightly regulated to prevent indiscriminant release of thrombogenic and inflammatory substances, growth factors, and surface expression of adhesion proteins. Regulation is achieved at several levels including control of *s*oluble *N*-ethylmaleimide-sensitive factor *a*ttachment *re*ceptors (SNAREs), proteins that provide the driving force for lipid membrane fusion, participation of the actin cytoskeleton, and changes in membrane phospholipids.

- Platelet granule release requires the fusion of granule membranes with surface-connected membranes (plasma membrane and OCS).
- SNAREs and their chaperones, cytoskeletal remodeling, and lipid modification all participate in membrane fusion required for platelet granule release.
- The dominant SNAREs that function in platelet granule secretion are VAMP-8, syntaxin-11, and SNAP-23.
- The exocytotic machinery interacts with cytoskeletal proteins: the cytoskeleton influences secretion and secretion is important for spreading.

SNAREs

SNAREs are membrane-associated proteins that are oriented to the cytosol (Fig. 2). SNAREs associated with granules are termed vesicular SNAREs (vSNAREs), while those

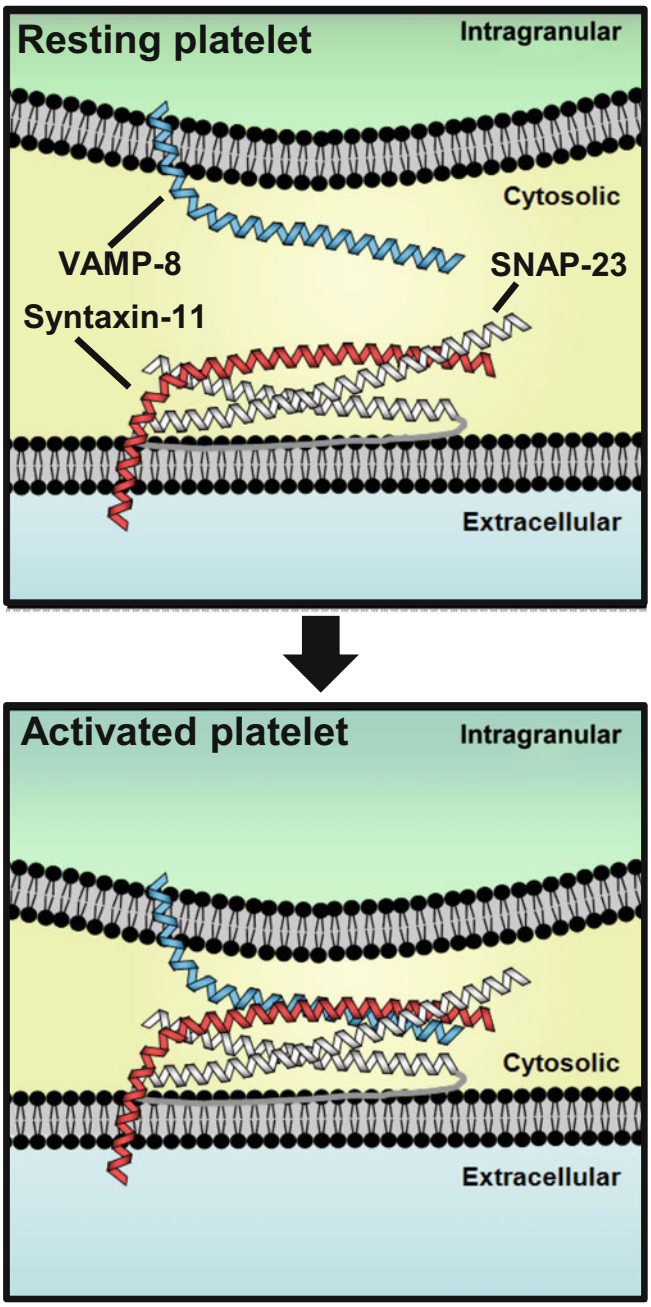

Fig. 2 Role of SNAREs in α-granule membrane fusion. (*Resting platelet*) VAMP-8 is a vSNARE found on platelet granules and is oriented with its N-terminus to the cytosol. Syntaxins-11 and SNAP-23 are tSNAREs found on plasma membranes and OCS and also oriented toward the cytosol. VAMP-8 and syntaxin-11 isoforms each contribute to one coiled-coil domain to the four-helix bundle while SNAP-23 contributes two. Only that portion of syntaxin that participates in the four-helix bundle is shown. (*Activated platelet*) vSNAREs (VAMP-8) and tSNAREs (syntaxin-11 and SNAP-23) initially associate at their N-terminal ends and interact via coiled-coil domains, bringing the opposing granule membrane and target membrane into close apposition. Binding of vSNAREs and tSNAREs generates energy required for membrane fusion (Adapted from Flaumenhaft 2013)

primarily associated with target membranes are termed tSNAREs. The characteristic feature of these proteins is the SNARE motif, a ~60 amino acid α-helix capable of interacting with other SNARE motifs to assemble into helical bundles involving coiled-coil interactions (Fig. 2). SNARE interactions occur in a parallel manner to form a four-helix bundle. Vesicle-associated membrane protein (VAMP) isoforms and syntaxin isoforms contribute one motif each and SNAP-23 contributes two motifs (Fig. 2). The binding of SNAREs brings granular and plasma membranes into close apposition. This tight association may also generate energy required for membrane fusion (Liu and Parpura 2010).

Platelets contain many SNARE isoforms. Known platelet vSNAREs include VAMP-2, -3, -4 -5, -7, and -8. Known platelet tSNAREs include syntaxins-2, -4, -7, -8, -11, and -12 and SNAP-23, -25, and -29 (Lemons et al. 1997; Flaumenhaft et al. 1999, 2007; Feng et al. 2001, 2002; Lai and Flaumenhaft 2003; Schraw et al. 2003; Ren et al. 2007; Dowal et al. 2011). However, it must be remembered that SNAREs participate in every level of membrane trafficking during megakaryopoiesis, so that only a subset of these platelet SNAREs function in granule secretion. VAMP-8- appears to be the dominant vSNARE involved in granule release (Fig. 2), since mice lacking VAMP-8 demonstrate a substantial (although not total) defect in granule release (Ren et al. 2007; Graham et al. 2009). VAMP-7 also functions in platelet granule release and may mediate coordination of granule release with cytoskeletal remodeling by virtue of an N-terminal extension that binds VPS9-ankyrin repeat protein (VARP) (Peters et al. 2012; Koseoglu et al. 2015b). VAMP-3 and perhaps VAMP-2 play subordinate roles (Schraw et al. 2003; Ren et al. 2007). Syntaxins 11 appears to be the dominant syntaxin isoform that functions in granule release (Fig. 2) based on the observation that both mice and humans that lack syntaxin 11 have a marked defect in granule release (Ye et al. 2012). A minor role for syntaxin 8 has recently been described (Golebiewska et al. 2014). The function of SNAP-23 in platelet granule release is well established (Fig. 2) (Flaumenhaft et al. 1999; Lemons et al. 2000; Polgar et al. 2003).

Posttranslational modification of SNAREs is thought to influence their activity. SNAP-23 is phosphorylated at Ser95 in an activation-dependent manner by IκB (Karim et al. 2013). Phosphorylation of SNAP-23 enhances membrane fusion in a SNARE-dependent liposome-mediated fusion system. Protein palmitoylation is a second posttranslational modification required for platelet granule release (Sim et al. 2007). VAMP-3, -4, -5, and -7; syntaxin-2, -8, -11, and -12; and SNAP-23 are all palmitoylated (Dowal et al. 2011). SNAP-23 is palmitoylated at a central membrane-binding domain, which is required for its association with membranes (Salaun et al. 2005).

SNARE Chaperones

Platelet SNAREs are regulated by a variety of binding proteins that direct their function. NSF is a Mg^{2+}-dependent hexameric ATPase that is essential for most forms of membrane trafficking, including regulated platelet granule secretion (Polgar and Reed 1999). Both inhibitory peptides and antibodies to NSF interfere with α-granule release from platelets (Polgar and Reed 1999). The *s*oluble *N*SF-*a*ttachment *p*rotein (SNAP), α-SNAP, binds and activates NSF (Clary et al. 1990). Wild-type α-SNAP augments platelet granule secretion, whereas antibodies directed at α-SNAP or a dominant-negative α-SNAP mutant (α-SNAPL294A) inhibit granule secretion (Chen et al. 2000). Syntaxin-binding proteins (STXBPs) are also important regulators of platelet granule secretion. STXBP2 (aka Munc18b) is a Sec/Munc protein that binds tightly to and regulates the activity of syntaxin-11 (Al Hawas et al. 2012). Platelets from patients with familial hemophagocytic lymphohistiocytosis type 5 (FHL5) lack STXBP2 and have defective granule secretion and a bleeding diathesis. STXBP5 (aka tomosyn) interacts with syntaxin-11/SNAP-23 heterodimers and mouse platelets lacking STXBP5 also demonstrate impaired granule release (Ye et al. 2014).

Rab proteins and their effectors are capable of docking opposing membranes and modifying SNARE function. Rab proteins represent the largest branch of the ras superfamily of GTPases and several of these, including Rabs 3b, 6c, and 8, are phosphorylated upon platelet activation (Karniguian et al. 1993). Rab GDP dissociation inhibitor (RabGDI), a general inhibitor of RabGTPases, inhibits α-granule but not dense granule release (Shirakawa et al. 2000). In addition, a dominant-negative mutant of His-tagged Rab4S22N (but not mutant His-Rab3BT36N) inhibits α-granule secretion but fails to affect dense granule release. These results suggest that Rab 4 is required for α-granule but not dense granule secretion. Rab27a localizes to lysosomal-related organelles and functions in dense granule release (Tolmachova et al. 2004, 2007). A mutation harbored by the *gunmetal* mouse in the α-subunit of the Rab geranylgeranyl complex prevents the attachment of geranylgeranyl to Rab proteins (Detter et al. 2000). Platelets from these mice demonstrate a defect in α-granule and dense granule secretion, as well as impaired granule synthesis. In nucleated cells, Rab proteins function by binding to large effector proteins that are proposed to interact with SNAREs directly or with proteins that regulate SNARE function (Zerial and McBride 2001). For example, Munc13-4 binds Rab27a in human platelets and mediates dense granule secretion (Ren et al. 2010). Patients with familial hemophagocytotic lymphohistiocytosis-3 (FHL-3) have mutated Munc13-4 and abnormal platelet granule release (Feldmann et al. 2003). In Jinx mice, which lack Munc13-4, dense granule release is essentially absent, while α-granule and lysosome release are also severely impaired (Ren et al. 2010). Synaptotagmin-like protein 4 (Slp4) interacts with Rab8 in platelets and mediates dense granule release (Hampson et al. 2012).

Cytoskeleton

The actin cytoskeleton is a dynamic structure that acts both as a barrier to prevent indiscriminant release of granule contents in resting platelets and as facilitator of membrane fusion and granule secretion in activated platelets. In resting platelets, F-actin coats purified platelet α-granules (Flaumenhaft et al. 2005). The barrier function of the actin cytoskeleton is evidenced by studies—using cytochalasins (Cox 1988), latrunculin A (Flaumenhaft et al. 2005), or the F-actin severing protein scinderin (Marcu et al. 1996)—that demonstrate increased dense granule release with inhibition of actin polymerization or with cleavage of F-actin. Inhibition of actin polymerization also augments the kinetics and degree of α-granule release (Flaumenhaft et al. 2005). These results indicate that some degree of F-actin disassembly might actually be required for normal granule secretion.

In contrast to the barrier function of the resting cytoskeleton, de novo actin polymerization during platelet activation contributes to granule release. High concentrations of inhibitors of actin polymerization block α-granule release (Woronowicz et al. 2010). In a cell-free platelet granule secretory system, inhibition of F-actin formation blocks release of α-granule contents, whereas actin polymerization stimulates α-granule release (Woronowicz et al. 2010). Actin polymerization may elicit membrane fusion by contributing to the transport or expulsion of granules or by distorting and destabilizing lipid bilayers (Eitzen et al. 2002). In addition, platelet SNAREs interact directly with actin components of the cytoskeleton. Perhaps the best example is the interaction of VAMP-7 with VARP (Fig. 3). VARP is an adaptor protein that functions in both granule exocytosis and cytoskeletal remodeling. Immunoprecipitation studies in platelets demonstrated an association of VAMP-7, VARP, and Arp2/3, an actin-binding protein that functions in actin polymerization (Koseoglu et al. 2015b). Deficiency of VAMP-7 impairs both platelet granule secretion and spreading (Koseoglu et al. 2015b). Similarly, GPS platelets, which lack α-granules, and platelets deficient in Munc13-4 also demonstrate impaired spreading (Peters et al. 2012), indicating a bidirectional link between granule exocytosis and platelet spreading. Recent studies evaluating spreading on micropatterned arrays indicate that platelet secretion responds to cues in the microenvironment to stimulate directional secretion (Sakurai et al. 2015). Directional secretion may, in turn, provide for deposition of new matrix on which platelet membranes can extend.

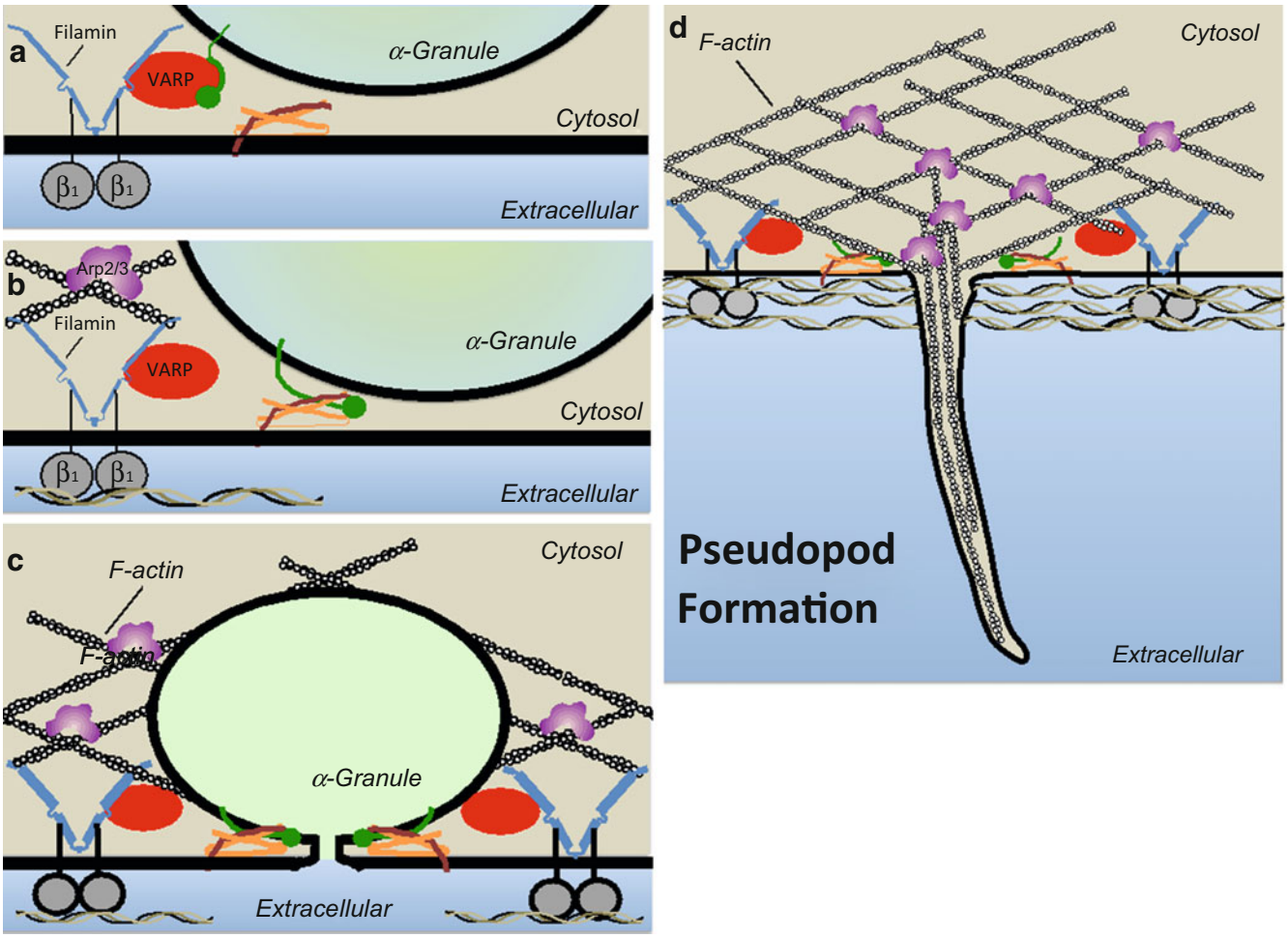

Fig. 3 A model of the molecular mechanisms linking platelet granule exocytosis and actin polymerization. (**a**) In the resting state, VAMP-7, which is associated with the granule membrane, binds to VARP, which associates with Arp2/3. This arrangement localizes the granule exocytosis machinery and the actin polymerization machinery to the same location and maintains them in an inactive state. (**b**) Mechanosensing via surface receptors (α2β1 shown here) is transmitted through actin-binding proteins (filamin A shown here) activating the platelet and releasing VAMP-7 and Arp2/3 from VARP. (**c**) VAMP-7 interacts with tSNAREs on the plasma membrane, facilitating membrane fusion, and Arp2/3 functions in actin reorganization. (**d**) Fusion of granules with the plasma membrane provides extra membrane to cover growing actin structures during spreading

Lipids

The stalk model of membrane fusion posits that release of contents from secretory cells occurs through a fusion pore (Kozlovsky and Kozlov 2002). This pore then expands, extruding granule contents and enabling the incorporation of granule membrane into plasma membrane. The fusion pore is short lived, however, and only recently have techniques such as single cell amperometry been developed with sufficient temporal resolution to provide experimental support for a fusion pore intermediate in platelets (Ge et al. 2008). Characterization of the fusion pore of platelet dense granules demonstrates a secretion time course of ~7 ms (Ge et al. 2008; Koseoglu et al. 2013). Dynamin family proteins that are capable of binding lipids and cleaving stalks of endocytotic and exocytotic vesicles have been invoked in platelet granule release.

Several phospholipid components of the platelet membrane influence its ability to fuse. Studies of fusion pore formation using single-cell amperometry demonstrate that cholesterol concentration influences fusion pore dynamics and granule release (Ge et al. 2008). The intrinsic negative curvature of cholesterol may stabilize intermediate membrane structures required for pore formation and expansion. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is synthesized in platelets in an activation-dependent manner by both type I and type II PIPKs (Tolias et al. 2000; Rozenvayn and Flaumenhaft 2001, 2003). A role for PIP_2 in platelet granule secretion has been demonstrated (Rozenvayn and Flaumenhaft 2001, 2003). Although the exact role of PIP_2

in granule release is not well understood, several SNARE regulating proteins and cytoskeletal proteins contain PIP_2-binding domains and may be recruited to sites of granule exocytosis by PIP_2 (McLaughlin et al. 2002). PIP_2 also serves as a precursor for diacylglycerol and IP_3, which are essential second messengers in stimulating protein kinase C (PKC) isoforms and Ca^{2+} flux. Phosphatidic acid (PA) synthesis correlates with granule secretion and inhibition of PA synthesis inhibits secretion (Coorssen and Haslam 1993). The role of PA in potentiating membrane fusion has not been defined. However, altering membrane curvature, serving as protein attachment sites, and signaling are proposed functions for PA in membrane fusion (Jones et al. 1999; Vitale et al. 2001). Phosphatidylserine enrichment in platelet membranes resulted in decreased platelet granule secretion (Koseoglu et al. 2015a). Despite the importance of lipid composition in membrane fusion, changes in lipid composition alone are not sufficient for regulated platelet granule secretion, which requires a protein-based fusion machinery.

Inheritable Defects of Platelet Granules

Much of what is known about the function of platelet granules has been learnt from patients with platelet granule deficiencies. Platelet granule defects are categorized as δ-storage pool deficiency in which dense granules are decreased, α-storage pool deficiency in which α-granules are decreased, and αδ-storage pool deficiency in which both granule types are decreased. Defects in platelet granule secretion despite normal granule content have also been described.

- Dense granule deficiencies are the most common platelet granule deficiency syndromes and are associated with a bleeding diathesis.
- α-Granule deficiency are typically associated with macrothrombocytopenia and mild-moderate bleeding.
- Granule deficiency syndromes have provided a wealth of information regarding the granulogenesis of dense granules and α-granules.
- BLOC proteins, AP-3, and Rab proteins mediate dense granule formation.
- Neurobeachin 2, VPS16B, VPS33B, and several transcription factors mediate α-granule formation.

Dense Granule Deficiencies

The most common and well-studied platelet dense granule defect occurs with Hermansky–Pudlak Syndrome (HPS). HPS patients have variable mucocutaneous bleeding and

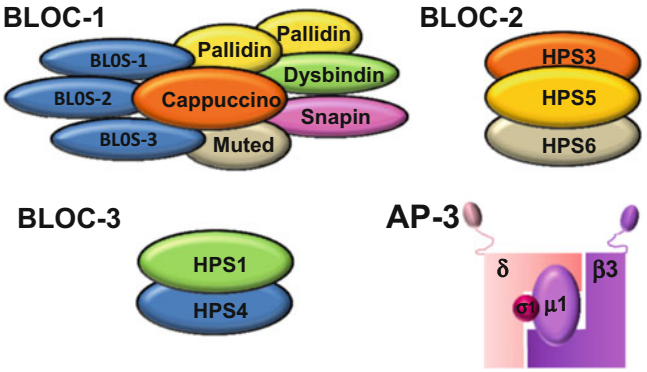

Fig. 4 Proteins that are defective in Hermansky–Pudlak syndrome. Biogenesis of Lysosome-related Organelle Complexes (BLOCs) function in the formation of dense granules. BLOC-1 contains 8 distinct proteins, with two copies of pallidin. BLOC-2 and BLOC-3 are composed of HPS protein subunits. AP-3 contains four subunits including δ, β3, μ1, and σ1. Mutations in genes encoding these proteins are responsible for HPS (Adapted from Flaumenhaft 2013)

significant postsurgical bleeding as a result of their platelet defect. In addition to the bleeding diathesis, patients suffer from oculocutaneous albinism resulting in impaired vision, inflammatory bowel disease, and progressive pulmonary fibrosis. Nine different variants of HPS have been described (Cullinane et al. 2011). Pulmonary fibrosis, a frequent cause of death in HPS, is most common in HPS-1 and HPS-4. The defect in dense granule formation results from mutations in HPS genes, which encode for components of BLOC and AP-3 complexes (Fig. 4). These complexes are required for the formation of lysosome-related organelles. Platelets from patients with HPS have a normal allotment of α-granules, suggesting that BLOC protein and AP-3 contribute to granulogenesis at a point distal to the sorting of α-granules from dense granules. However, release of α-granules and lysosomes from HPS platelets is also impaired (Meng et al. 2015). In addition, the release of protein disulfide isomerase (PDI) (Sharda et al. 2015), which is stored in T-granules derived from the dense tubular system (Thon et al. 2012), is also impaired in HPS. PDI serves an essential role in thrombus formation and impairment of its secretion contributes to defective thrombosis in HPS (Sharda et al. 2015).

Several other syndromes have been described that include dense granule deficiency. Chediak–Higashi syndrome (CHS) is an autosomal recessive disorder caused by mutations in the *LYST* gene (Barbosa et al. 1996; Perou et al. 1996). Platelets from these patients lack dense granules. CHS patients suffer from immunodeficiency, albinism, a bleeding diathesis, and neurological disorders. Many of the patients die from a lymphoproliferative syndrome. Bleeding in Chediak–Higashi syndrome patients is typically mild to moderate. Patients with Griscelli syndrome type II have a defect in Rab27a and can have bleeding along with dense granule deficiency. However, platelets also express

Rab27b, which largely compensates for the defect in Rab27a (Barral et al. 2002). Patients with thrombocytopenia with absent radii (TAR) syndrome are diagnosed on the basis of a deleted 200-kb region at chromosome band 1q21.1 and can have decreased dense granules (Klopocki et al. 2007). Wiskott–Aldrich syndrome results from a mutation in WASp, which regulates actin filament organization in complex with actin binding and nucleating proteins, and is also associated with decreased dense granules (Derry et al. 1994). Bleeding has been associated with all these dense granule deficiency syndromes.

α-Granule Deficiencies

The best known inherited disorder of α-granule formation is Gray platelet syndrome (GPS). Clinical features of GPS include progressive macrothrombocytopenia, bone marrow fibrosis, and splenomegaly. The bleeding diathesis has been defined as mild to moderate. Severe bleeding is observed almost exclusively in women with menorrhagia (Gunay-Aygun et al. 2010). Autosomal recessive GPS results from mutations in *NBEAL2* (Albers et al. 2011; Gunay-Aygun et al. 2011; Kahr et al. 2011). *NBEAL2* encodes for neurobeachin 2, a member of a family of proteins containing Beige and Chediak Higashi (BEACH) domains. These domains were originally identified in LYST, the protein that is mutated in Chediak–Higashi Syndrome. Neurobeachin 2 functions in granule trafficking. Autosomal positive GPS is caused by a mutation in growth factor independent 1B (GFI1B) resulting in a dominant negative form of this transcription factor (Monteferrario et al. 2014).

ARC syndrome is also associated with platelet α-granule deficiency. ARC syndrome is caused by mutations in a protein, VPS33B, which is involved in vesicle trafficking (Gissen et al. 2004; Lo et al. 2005). VPS33B is a membrane-associated protein that binds tightly to and regulates the function of SNAREs. In platelets, VPS33B associates with α-granules (Lo et al. 2005). Patients with this mutation possess α-granule-deficient platelets, and their platelets possess no detectable CXCL4, vWf, fibrinogen, nor P-selectin (Lo et al. 2005). Thus, loss of VPS33B affects incorporation of both endogenous and endocytosed proteins, as well as both soluble and membrane-bound proteins, into α-granules (Lo et al. 2005). The number of dense granules in VPS33B-deficient platelets is modestly increased, indicating that VPS33B function is not critical for dense granule formation. Platelets from mice with an inducible deficiency in VPS33B demonstrate a reduction in mature type II multivesicular bodies (MVB II), indicating that VPS33B functions in the maturation of α-granules as they are formed from multivesicular bodies (Bem et al. 2015).

In the Quebec platelet disorder, α-granule proteins are degraded by intragranular plasmin, which is present owing to upregulation of urokinase plasminogen activator in megakaryocytic α-granules (Veljkovic et al. 2009). The increase in megakaryocyte urokinase plasminogen activator activity is secondary to duplication of *PLAU*, the gene that encodes this protease (Paterson et al. 2010). Quebec platelet disorder is associated with a bleeding diathesis (Hayward et al. 1997).

Mutations in the transcription factor, RUNX1, result in several platelet abnormalities including CXCL4 deficiency and α-granule cargo abnormalities (Aneja et al. 2011). Mutations in GATA1 have been described in patients with thrombocytopenia and markedly reduced or absent α-granules (Balduini et al. 2004; Tubman et al. 2007).

Defects in Granule Release

Familial Hemophagocytic Lymphohistiocytosis (FHL) is a family of genetic disorders originally characterized on the basis of immunodeficiency resulting from impaired exocytosis of cytotoxic granules from natural killer cells and cytotoxic T lymphocytes (Tang 2015). Several forms of FHL are associated with a bleeding diathesis owing to impaired secretion of platelet granules. Several variants of FHL exist and the causative genetic defects have been identified. FHL-3 results from mutations in Munc13-4, which is critical for granule exocytosis in mice and men (Feldmann et al. 2003). FHL-4 results from mutations in syntaxin 11 (zur Stadt et al. 2005). FHL-5 results from mutations in Munc18b (zur Stadt et al. 2009). A severe defect in platelet dense and α-granule secretion has been reported in platelets from patients with FHL-5 (Sandrock et al. 2010).

Conclusion

Since the identification of the platelet granule types—dense granules, α-granules, and lysosomes—by electron microscopy over half a century ago, substantial progress has been made in our understanding of how platelet granules are made, what they contain, and how their contents are released upon platelet activation. Yet despite this progress, fundamental issues remain unresolved. The precise nature of α-granules, whether they constitute a single homogenous granule type or subpopulations that can be distinguished on the basis of morphology or cargo content remains controversial. New imaging technology such as superresolution microscopy, videomicroscopy, and electron tomographic analysis of platelet morphology will enhance our ability to decipher the complex nature of fusion events that include

fusion of granule membranes with plasma membrane, OCS, and other granules and may help identify granule subpopulations. While much progress has been made in identifying the SNARE isoforms and auxiliary proteins that mediate platelet membrane fusion, an understanding at the molecular level of how this machinery responds to activation signals such as elevation of intracellular Ca^{2+} and protein phosphorylation and dephosphorylation have only begun to be evaluated. Most previous studies of platelet secretory mechanisms have evaluated suspension platelets, neglecting the fact that physiological secretion occurs in adherent platelets and that the cytoskeleton has a critical influence on membrane fusion events involved in secretion. The role of mechanosensing and cytoskeletal elements in mediating granule secretion and the bidirectional interactions between exocytosis and cytoskeletal remodeling are only beginning to be understood. Understanding the molecular basis of granule secretion will enable development not only of new antiplatelet agents for use in thrombosis, but potentially for the treatment of many other diseases in which platelet granules have been invoked, such as inflammation, tumor progression, and atherosclerosis.

Take Home Messages

- Dense granules and α-granules are lysosome-related organelles found only in platelets.
- Both dense granules and α-granules are required for normal hemostasis and thrombosis.
- Dense granules contain primarily bioactive small molecules and cations.
- α-Granules contain a matrix of tightly packed and varied proteins that participate in a broad range of physiological activities.
- Lysosomes contain enzymes for degrading proteins, carbohydrates, and lipids.

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A Systems Approach to the Platelet Signaling Network and the Hemostatic Response to Injury

Lawrence F. Brass, Peisong Ma, Maurizio Tomaiuolo, Scott L. Diamond, and Timothy J. Stalker

Abstract

An essential part of the hemostatic response to injury is a well-calibrated accumulation of activated platelets. Here we will consider, first, how individual signaling and regulatory pathways in platelets combine to produce a flexible signaling network that can respond appropriately to injury under conditions that range from the macro- to the microcirculation and, second, how the piling up of platelets at a site of injury reshapes the local environment in ways that help to limit continued platelet accumulation. Using a systems biology perspective, we will examine the interrelationships between the platelet signaling network, the distribution of platelet agonists, and thrombus structure. The model that this produces moves beyond the behavior of any single platelet to consider the emergent properties of the platelet mass, thereby helping to explain why a multiplicity of agonists and signaling pathways are needed to produce an optimal hemostatic response.

Why a Systems Perspective?

Systems biology can be defined in a variety of ways. In a narrow sense, it combines computational approaches and mathematical modeling to understand biology. In the broader sense that we will use here, systems biology is the study of complex interactions whose properties become apparent only when multiple cells and multiple pathways are considered. The biological system in this case is the hemostatic response to injury. The cells are platelets and the pathways are those that either promote or limit platelet activation in response to the combined effects of multiple agonists and antagonists. Earlier chapters in this volume and previous reviews (Versteeg et al. 2013; Brass et al. 2013b) have focused on the individual receptors and pathways that are central to platelet activation. Here we will take a step back from considering events in any one pathway or platelet, and instead consider how activation and regulatory pathways within platelets integrate extracellular cues, forming a flexible signaling network capable of supporting, shaping, and limiting the hemostatic response.

Viewed from this broader perspective, platelets are small cells with the ability to shift rapidly from a freely moving state in which they are normally quiescent to an adherent and activated state, acquiring in the process the ability to form a temporary barrier against further blood loss. In this perspective, vascular injuries trigger platelet accumulation by the local exposure and production of platelet agonists, some of which are stationary (collagen), while others are mobile (thrombin, ADP, TxA_2, and epinephrine). Soluble molecules are able to achieve the critical concentrations needed for platelet

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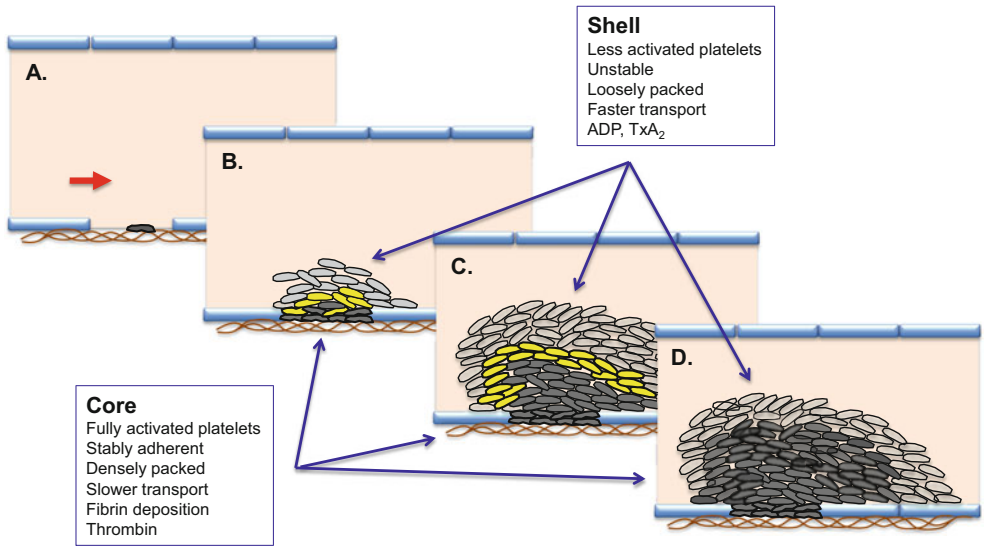

Fig. 1 Development of the heterogeneous architecture observed within hemostatic thrombi. Over time, hemostatic thrombi formed in response to penetrating injuries pass through the stages illustrated here, developing a characteristic structure in which fully activated platelets in a tightly packed core (*dark gray*) are overlaid with a more loosely packed shell (*light gray*) of incompletely activated platelets. A boundary region of higher packing density platelets that have not yet become fully activated is initially present at the boundary between the core and shell regions (*yellow*). Detectable thrombin activity and fibrin accumulation are limited to the core. In the process, individual platelets become increasingly surrounded by other activated platelets, radically altering their immediate environment

activation only if they are produced faster than they are washed away or inhibited. As will be discussed, the ability of soluble platelet agonists to accumulate is due in part to the protection provided by the growing platelet mass (Fig. 1). As a result, the platelet signaling network and thrombus structure are interdependent, possessing properties that cannot be fully understood by considering any one pathway or any one platelet at a time.

While platelet behavior has been observed for well over a century, advances in intravital imaging pioneered by the Furie laboratory (Falati et al. 2002, 2004; Celi et al. 2003) and others (oude Egbrink et al. 1988; Rosen et al. 2001; van Gestel et al. 2002; Stalker et al. 2013) have made it possible to observe the hemostatic response to penetrating injuries in real time at high resolution in mouse models. Interpretation of the results benefits from a systems perspective, which includes considering the ways that molecular transport changes in the gaps between platelets as platelets accumulate, the gaps grow tortuous, and the hemostatic mass continues to grow. The imaging studies show that platelet activation within a hemostatic thrombus is heterogeneous but not random. While some platelets change shape, activate their integrins, secrete their granule contents, and develop a procoagulant surface, others display few, if any, signs of activation. Observational studies show that this heterogeneity is ordered, producing a core of fully activated platelets closest to the site of injury that is overlaid with a shell of less activated platelets (Fig. 2) (Stalker et al. 2013).

Among the properties that distinguish the thrombus core from the shell is platelet packing density, which is greater in the core than the shell (Figs. 1 and 2) (Stalker et al. 2013). Greater packing density slows the movement of plasma-borne molecules and platelet-derived molecules passing through the gaps between platelets. Soluble molecules can include platelet agonists and coagulation factors, as well as coagulation inhibitors, inflammatory response mediators, and any other molecule found in plasma. Computational studies predict and experimental studies demonstrate that transport rates are slower in the core than in the shell and, especially in the most densely packed regions, are dominated by diffusion rather than convection due to permeation (Stalker et al. 2014; Tomaiuolo et al. 2014; Welsh et al. 2014). Regional transport differences affect the distribution of platelet agonists, producing concentration gradients in which the distribution of each agonist is determined by factors such as size, shape, half-life, and ability to bind to other molecules, including those present on the platelet surface. Greater packing density also brings platelets into closer contact with each other, enabling direct interactions between paired molecules on adjacent platelet surfaces (Brass et al. 2005).

Thus, part of the systems perspective on the hemostatic response to injury is that as platelets accumulate, the immediate environment of each platelet changes in ways that impact the growth and stability of the entire hemostatic mass. In this chapter, we will briefly consider how the platelet signaling network responds to combinations of agonists, how thrombus architecture both reflects and determines the distribution of those agonists, and how the timing of inhibitory events within each platelet helps shape as well as limit the hemostatic response.

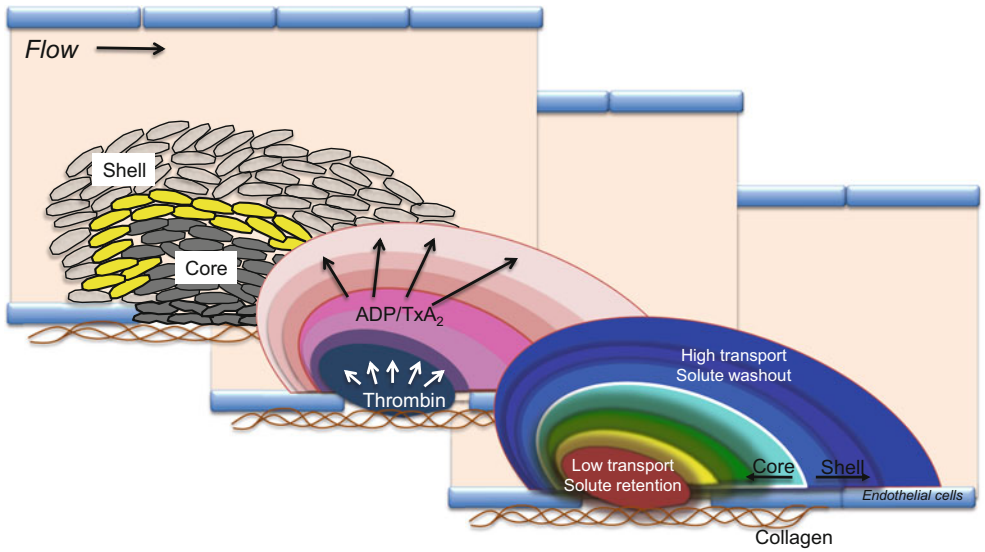

Fig. 2 The relationships between packing density, transport rates, agonist concentration gradients, and thrombus structure. As platelets within a developing thrombus become activated, they change shape, retract, and pack tightly together, driving the formation of regions with lower and higher transport rates (*right*). This leads to the retention of larger agonists, such as thrombin, within the low transport region. Smaller agonists, such as ADP and TxA_2, are able to diffuse more freely out of the low transport region (*middle*). The localization of these agonist gradients drives continued platelet activation in the low transport region leading to core formation consisting of high platelet packing density, decreased solute transport, α-granule exocytosis, and fibrin deposition (*left*). The restriction of thrombin to the core contributes to the shell consisting of loosely adherent platelets, high solute transport, reduced platelet activation, and no fibrin. Adapted from Welsh et al. (2014)

Platelet Activation Reflects Information Flow Through a Signaling Network

Figure 3 illustrates the platelet signaling network leading to integrin activation, stripped to its essentials for the purpose of this discussion and emphasizing the on/off cycling of heterotrimeric G proteins, Rap1b, and adenylate cyclase. When platelets respond to injury in vivo this network is called upon to sum the response to multiple platelet agonists and inhibitors located near each platelet, including collagen, thrombin, ADP, TxA_2, epinephrine, NO, and PGI_2, not all of which may be present at optimal or sustained concentrations.

Critical events in this network include the activation of phospholipase C (PLC), an increase in cytosolic Ca^{++}, reorganization of the platelet cytoskeleton, granule secretion, and the steps that link Ca^{++} to activation of the platelet fibrinogen receptor, $\alpha_{IIb}\beta_3$, so that platelet aggregates can form. Critical inhibitory events include an increase in cAMP and cGMP levels, driven by the local concentrations of PGI_2 and NO, and the subsequent phosphorylation of multiple platelet proteins. Most of the agonists and antagonists listed (collagen and NO are two exceptions) signal through G protein-coupled receptors on the platelet surface, producing distinctive responses by coupling to different repertoires of heterotrimeric G proteins. As an example, collagen, thrombin, and TxA_2 all cause phosphoinositide (PI-4,5-P_2) hydrolysis, IP_3 formation, and a subsequent rise in the cytosolic Ca^{++} concentration, but collagen accomplishes this by activating phospholipase Cγ via a tyrosine kinase-dependent mechanism downstream of glycoprotein VI, while thrombin and TxA_2 do so via Gq-dependent activation of phospholipase Cβ downstream of protease-activated receptors (PAR1 and PAR4 in human platelets) and TxA_2 receptors (TP) (see earlier chapters in this volume) (Stalker et al. 2012; Versteeg et al. 2013; Brass et al. 2013a, b).

Crosstalk between distinct pathways can amplify responses, as when thrombin promotes Rap1b activation via a Ca^{++} -dependent guanine nucleotide exchange factor, CalDAG-GEF (Crittenden et al. 2004), while ADP (and presumably epinephrine) promote Rap1b activation by inhibiting the turn-off of Rap1b by RASA3 in a PI-3 kinase (PI3K)-dependent manner (Stefanini et al. 2015). Rap1b is linked to integrin activation in platelets via RIAM and talin (Shattil et al. 2010). Positive autocrine and paracrine feedback loops occur when agonists activate phospholipase A_2, releasing arachidonate from membrane phospholipids to undergo cyclooxygenase-1 (COX-1)-dependent conversion to TxA_2.

At the same time, extrinsic regulators of platelet activation, including PGI_2 and NO, dampen platelet responsiveness and resist unwarranted platelet activation, while intrinsic regulators, such as RGS (Regulators of G protein Signaling) proteins, limit signaling downstream of platelet agonists once it has been initiated. RGS proteins limit the duration of G protein signaling by accelerating

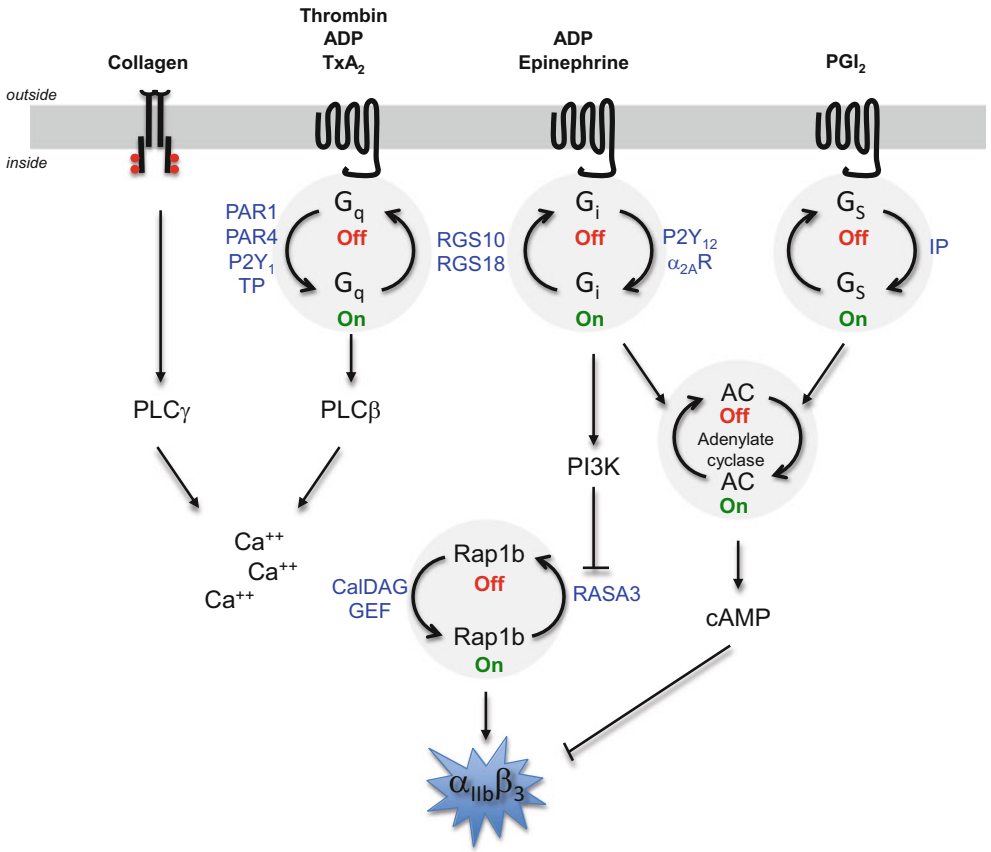

Fig. 3 The platelet signaling network leading to integrin $\alpha_{IIb}\beta_3$ activation: switching events, crosstalk, and regulatory feedback. A view of the platelet signaling network connecting platelet agonists to the platelet cohesion receptor, $\alpha_{IIb}\beta_3$, stripped to its essentials and emphasizing the role of switching events, crosstalk between pathways, and regulatory events. Platelet agonists activate either phospholipase Cγ (collagen) or phospholipase Cβ (thrombin, ADP, and TxA_2) raising IP_3 levels and triggering a rise in the cytosolic Ca^{++} concentration. Increased Ca^{++} activates the exchange factor, CalDAG-GEF, activating the monomeric G protein, Rap1b, which is linked to integrin activation by RIAM and talin (see text). Rap1b activation is also promoted by ADP via G_i, which promotes Rap1b signaling by inhibiting the Rap1b inhibitor, RASA3 (Stefanini et al. 2015). The switching events in this case center on Rap1b and the heterotrimeric G proteins, G_q and G_i. Regulatory events include the inactivation of G_q and G_i by RGS10 and RGS18, the inactivation of Rap1b by RASA3. cAMP dampens platelet responsiveness at a number of places in the platelet signaling network, including receptor desensitization, inhibition of IP_3 receptors, and an increase in free RGS18

the return of activated G proteins to the off state. Platelets express predominantly RGS10 and RGS18 (Rowley et al. 2011; Burkhart et al. 2012), which appear to be sequestered in resting platelets by the scaffold proteins, spinophilin (Ma et al. 2012) and 14-3-3γ (Gegenbauer et al. 2012) (Fig. 4). In the case of spinophilin, the release of RGS10 and RGS18 that occurs during platelet activation is agonist selective (Ma et al. 2012). Such selectivity provides a mechanism for fine tuning the consequences of platelet activation as information flow is restricted in some pathways but not others. PGI_2 and NO reduce the reactivity of resting platelets to most, if not all agonists by raising intracellular cAMP and cGMP levels, activating protein kinases A and G, and causing phosphorylation of multiple proteins in the platelet signaling network, including G protein-coupled receptors and the IP_3 receptor (Quinton and Dean 1992; Grunberg et al. 1995a, b; Smolenski 2011; Margarucci et al. 2011; Wangorsch et al. 2011; Gambaryan et al. 2010; Hayashi and Sudo 2009; Zahedi et al. 2008; Gegenbauer et al. 2012). In an example of crosstalk, cAMP dampens signaling in part by causing the dissociation of RGS proteins from spinophilin (Ma et al. 2015a, b) and 14-3-3γ (Gegenbauer et al. 2013), thereby reducing signaling downstream of platelet agonist receptors. Such events help to mold the growing thrombus by limiting the extent of platelet activation.

In a systems and network-centric view of platelet activation, instead of responding to a single agonist via a single signaling pathway, platelets accumulating at a site of injury respond to the local mix of inputs (agonists and antagonists) to which they are exposed. In vivo this mix varies over time and space, which is especially relevant when considering that the impact of multiple agonists can be synergistic and

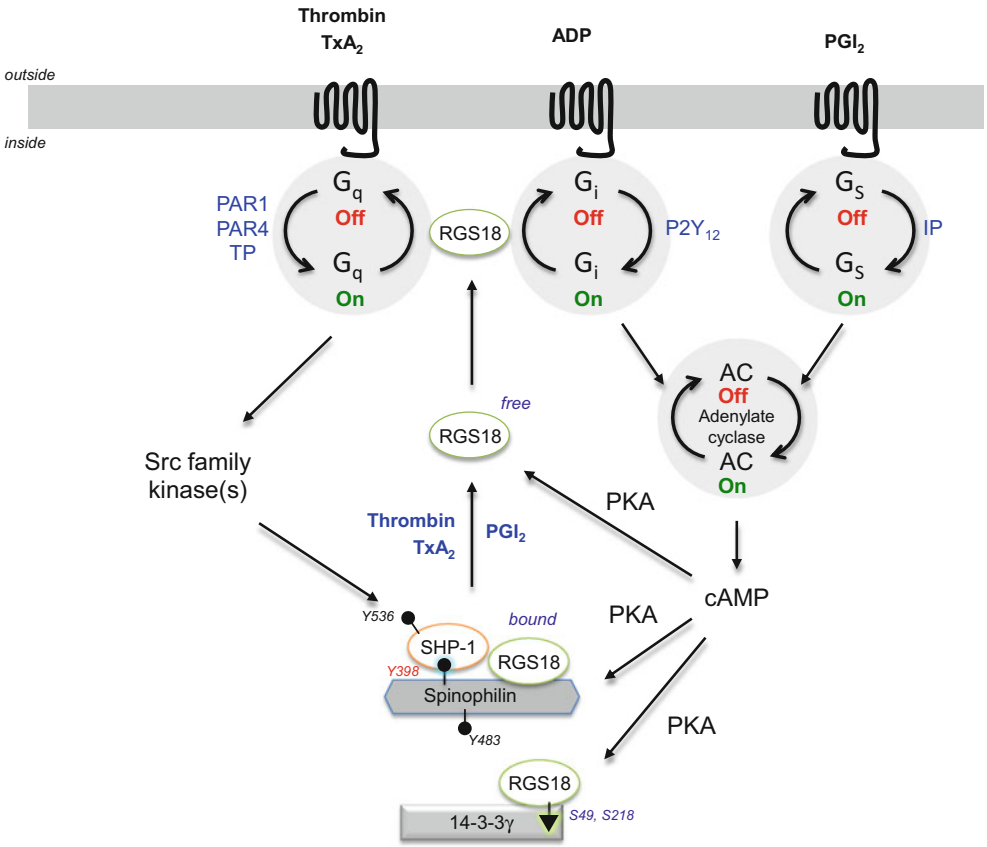

Fig. 4 RGS proteins as regulators of the platelet signaling network. In resting platelets, RGS18 is part of separate complexes built on the scaffold proteins, spinophilin, and 14-3-3γ. Addition of thrombin or TxA_2 causes dissociation of the SPL/RGS18/SHP-1 complex, freeing RGS18. Addition of PGI_2 to raise platelet cAMP levels has much the same effect, although by a different mechanism. Rising cAMP levels activate protein kinase A (PKA), which phosphorylates spinophilin and RGS18, causing dissociation of RGS18 from its binding site on 14-3-3γ as well as spinophilin. An increase in free RGS18 dampens signaling mediated by G_q and G_i in platelets. Comparable events are presumed to regulate RGS10 availability, although that has not been studied to the same extent

not simply additive (Chatterjee et al. 2010; Lee and Diamond 2015; Flamm et al. 2012). Positive and negative feedback in the form of soluble molecules plus contact-dependent interactions between adjacent platelets plays an important role in modulating platelet reactivity. Information flow within the platelet sums the effects of these inputs while nodal points in the signaling network provide links between them. As will be discussed later in this chapter, this view of platelet activation can be tied to thrombus architecture by considering, first, how gradients of different platelet agonists emanating from the point of injury differentially impact the platelet activation state and, second, how the higher packing density of platelets in the thrombus core facilitates the binding of cell surface ligands to cell surface receptors on adjacent platelets (Brass et al. 2005). These properties emerge only when considering the differences between single platelets in isolation and a multiplicity of platelets in close proximity with one another.

Studies of platelet activation performed in vitro in the clinical and research settings are commonly limited to the impact of a few agonists added one at a time in a closed, well-mixed system with concentrations that do not vary over time. Agonist concentration gradients do not form. Endpoints are often limited to platelet aggregation and secretion, supplemented in the research setting by flow cytometry using activation markers, measurements of cytosolic Ca^{++}, and accumulation on collagen and tissue factor coated surfaces in flow chambers and microfluidics devices. With the exception of the flow chambers and microfluidics devices, platelet activation in each of these settings occurs under conditions very different from those encountered in vivo. A good example of the consequences of exposure to more than one agonist comes from studies in which signaling initiated by exposure to both thrombin and collagen results in a sustained rise in the cytosolic Ca^{++} concentration, which in turn greatly promotes platelet procoagulant activity (Keuren et al. 2005).

Higher dimensional combinatorial approaches can be used to more closely mimic and predict what happens to individual platelets located within a thrombus. One approach is to expose platelets in vitro to as many as possible combinations of the agonists and inhibitors that are encountered in vivo: collagen (or a soluble GP VI agonist, such as convulxin), thrombin (or peptide agonists for PAR1 and PAR4), ADP, TxA_2 (or its stable mimetic, U46619), PGI_2 (or iloprost), and NO. Given sufficiently high throughput, each agonist and antagonist can be added at its mid-point concentration (determined from dose/response curves) as well as at concentrations that produce minimal and maximal responses. Biologically relevant readouts that have been

studied in this way include cytosolic Ca^{++} (Chatterjee et al. 2010; Lee and Diamond 2015), $\alpha_{IIb}\beta_3$ activation, and α-granule exocytosis (Jaeger and Diamond 2013). The data obtained with combinations of 1, 2, or 3 agonists and inhibitors have been used to train neural networks that correctly predict the synergistic effects of agonist combinations and the effects of combining even greater numbers of agonists. They have also been used to define differences among healthy individuals and to identify an unsuspected defect in TxA_2 signaling due to an amino acid substitution within the platelet TxA_2 receptor (Chatterjee et al. 2010; Lee and Diamond 2015; Flamm et al. 2012).

Network Regulation

The molecular mechanisms that drive platelet activation reflect an evolutionary compromise. This compromise can be thought of as establishing a threshold for platelet activation. If the threshold is too high, then platelets become useless for hemostasis. If too low, then the risk of thrombosis increases. An optimal platelet response to a penetrating injury can be defined as one in which hemostasis is achieved without unnecessary vascular occlusion. The set point is established by balancing signaling mechanisms that drive platelet activation with regulatory mechanisms that either dampen those responses or prevent their initiation in the first place. The normal environment of the platelet includes other blood cells, the soluble molecules found in plasma and, most critically, the vascular wall and the endothelial cells that line it, all of which are subject to change in the face of injury, disease, circadian rhythms, and aging. Many of these serve as extrinsic regulators of platelet activation. A healthy endothelium provides a physical barrier that limits platelet activation. It also produces inhibitors of platelet activation including NO (Furlong et al. 1987), prostacyclin (PGI_2) (Whittle and Moncada 1983; Weksler 1982; Yuhki et al. 2011), and the ecto-ADPase, CD39, which hydrolyzes plasma ADP that would otherwise sensitize platelets to activation by other agonists (Marcus et al. 1997).

In addition to these extrinsic regulators, there are intrinsic regulators that affect platelet activation. These include protein tyrosine phosphatases such as SHP-1; the cyclic nucleotides, cAMP and cGMP, which inhibit signaling events by the allosteric effects of protein serine and threonine phosphorylation; and RGS proteins and their binding partners, which limit signaling downstream of G protein-coupled receptors in platelets. Here we will focus on cAMP and RGS proteins since they provide two strong examples of network regulation and, in addition, illustrate how crosstalk between regulatory pathways can provide a context-dependent means of regulating platelet reactivity.

Regulation of G Protein-Dependent Signaling

Most platelets agonists activate platelets by binding to G protein-coupled receptors on the platelet surface. Because mechanisms exist that can limit the activation of this class of receptors, platelet activation can be tightly regulated. Receptor internalization and desensitization provide two such mechanisms. Another is provided by members of the RGS (Regulator of G protein Signaling) protein family, which limit signaling intensity and duration by accelerating the hydrolysis of GTP by activated G protein α subunits and allowing Gα to reassociate with Gβγ (Hollinger and Hepler 2002; Sjogren and Neubig 2010; Siderovski and Willard 2005). There are 37 genes encoding RGS proteins in the human genome. Transcripts for at least 8 have been detected in platelets, but protein studies suggest that human and mouse platelets express predominantly RGS10 and RGS18 (Ma et al. 2012; Rowley et al. 2011; Burkhart et al. 2012; Zeiler et al. 2014). Both proteins are relatively small, consisting primarily of a conserved RGS domain that interacts with Gα (Kimple et al. 2011). Each can serve as a GAP (GTPase activating protein) for Giα and Gqα but not Gsα (Soundararajan et al. 2008; Hunt et al. 1996; Popov et al. 1997; Nagata et al. 2001; Gagnon et al. 2002). RGS18 is primarily expressed in hematopoietic cells (Nagata et al. 2001; Park et al. 2001; Yowe et al. 2001; Gold et al. 1997; Hunt et al. 1996); RGS10 is widely expressed (Yang and Li 2007; Lee et al. 2008, 2011).

The evidence that RGS proteins are biologically relevant in platelets comes from two recent studies on RGS18 knockout mice (Delesque-Touchard et al. 2014; Alshbool et al. 2015) and on mice in which glycine 184 in the α subunit of G_{i2} has been replaced with serine (Signarvic et al. 2010). The G184S substitution prevents $G_{i2}\alpha$ from binding to RGS proteins without impairing the ability of G_{i2} to interact with either receptors or effectors (Fu et al. 2004). G_{i2} is the G protein that couples platelet $P2Y_{12}$ receptors to Akt and Rap1b and to the suppression of cAMP formation by adenylyl cyclase (Fig. 3). The knockout of the gene encoding $G_{i2}\alpha$, like the $P2Y_{12}$ receptor knockout, impairs platelet responses to ADP and produces a loss of function in vitro and in vivo (Jantzen et al. 2001; Hollopeter et al. 2001; Yang et al. 2002). The G_{i2} G184S substitution produces a gain of function in vitro and in vivo, increasing responsiveness to some platelet agonists, and enhancing thrombosis in mouse models of induced thrombosis without causing overt spontaneous thrombosis (Signarvic et al. 2010; Brass et al. 2013b). Similar effects have been reported with the RGS18 knockout (Delesque-Touchard et al. 2014; Alshbool et al. 2015). The effects observed with the RGS18 knockout suggest that RGS10 and RGS18 may not be fully redundant in platelets, but this conclusion awaits studies on platelets lacking RGS10.

If RGS proteins serve as brakes, how is the application of the brakes regulated so that platelet activation can proceed when needed? Several observations suggest that RGS10 and RGS18 are at least partially sequestered in resting platelets. Both RGS proteins have been shown to bind to the scaffold protein, spinophilin (neurabin-II or SPL) in resting platelets (Ma et al. 2012). RGS18 has additionally been shown to bind to the 14-3-3 family member, 14-3-3γ (Gegenbauer et al. 2012). The association and dissociation of the RGS proteins with each of these partners involves different mechanisms. In resting platelets, RGS10 and RGS18 form a complex with spinophilin that includes the tyrosine phosphatase, SHP-1. Within this complex, spinophilin is phosphorylated on tyrosine residues Tyr398 and Tyr483, with phosphorylated Tyr398 providing a binding site for SHP-1 (Ma et al. 2012). Platelet activation by thrombin activates SHP-1. This triggers dephosphorylation of spinophilin and the gradual decay of the SPL/RGS/SHP-1 complex that takes about 1 min to complete when measured in vitro. At least in the case of thrombin, dissociation of the complex is due to phosphorylation of SHP-1 Tyr536 by members of the Src family of tyrosine kinases, activating the phosphatase (Fig. 4). Binding to 14-3-3γ, on the other hand, is mediated by phosphorylation of RGS18 Ser49 and Ser218. Ser49 phosphorylation increases during platelet activation (Gegenbauer et al. 2012). Binding to 14-3-3γ inhibits the interaction of RGS18 with activated G proteins (Gegenbauer et al. 2013). Collectively, these observations suggest a model in which RGS10 and RGS18 provide a brake on excessive platelet activation, with the availability of the RGS proteins subject to somewhat divergent interactions with spinophilin and 14-3-3γ (Fig. 4). Studies on spinophilin knockout mice are consistent with this hypothesis (Ma et al. 2012), as are results obtained with an assay for free RGS18 in platelets (Ma et al. 2015a).

cAMP and Protein Kinase A

Rising cAMP levels inhibit platelet reactivity, rendering them resistant to activation by any of the agonists that they subsequently encounter. Thus, drugs that raise platelet cAMP levels have clinically useful antiplatelet activity (Patrono et al. 2008; Jackson and Schoenwaelder 2003) and, conversely, knocking out platelet PGI_2 receptors in mice produces a prothrombotic phenotype (Murata et al. 1997). When added to platelets in vitro, PGI_2 causes a tenfold rise in the platelet cAMP concentration, but even a relatively small increase (twofold or less) impairs platelet activation (Keularts et al. 2000). Deletion of either $G_{i2\alpha}$ or $G_{z\alpha}$ causes an increase in the basal cAMP concentration in mouse platelets (Yang et al. 2002). cAMP phosphodiesterase inhibitors such as dipyridamole act as antiplatelet agents by raising cAMP levels. Conversely, loss of PGI_2 receptor (IP) expression causes a decrease in basal cAMP levels, an enhanced response to agonists, and a predisposition to thrombosis in murine arterial injury models (Yang et al. 2002; Murata et al. 1997). Despite ample evidence that cAMP inhibits platelet activation, the mechanism by which it does this is not fully understood. cAMP-dependent protein kinase A is clearly involved, but other mechanisms may be as well. Substrates for PKA in platelets include GPCRs, IP_3 receptors, GPIbβ, vasodilator-stimulated phosphoprotein (VASP), and Rap1b (Quinton and Dean 1992; Grunberg et al. 1995a, b; Smolenski 2011; Margarucci et al. 2011; Wangorsch et al. 2011; Gambaryan et al. 2010; Hayashi and Sudo 2009; Zahedi et al. 2008; Gegenbauer et al. 2012) and, as it turns out, RGS18 (Gegenbauer et al. 2013) and spinophilin (Ma et al. 2015a).

Crosstalk Among Network Regulators

As was described earlier, current evidence shows that spinophilin and 14-3-3γ are able to sequester RGS proteins in resting platelets. Gradual dissociation of the SPL/RGS/SHP-1 complex when platelets are activated can potentially lead to an increase in free RGS10 and RGS18, but does this actually occur? Although the RGS18 and spinophilin knockout studies support this conclusion, the evidence has been indirect until recent studies showing that free RGS18 levels increase when platelets are incubated with either a thrombin receptor agonist peptide, or with PGI_2 and forskolin, both of which cause an increase in platelet cAMP levels (Ma et al. 2015a).

These results provide an example of crosstalk between inhibitory pathways within the platelet signaling network. The increase in free RGS18 levels occurs for at least two reasons. cAMP-dependent phosphorylation of RGS18 on Ser216 causes it to dissociate from 14-3-3γ (Gegenbauer et al. 2013). cAMP-dependent phosphorylation of spinophilin Ser94 causes dissociation of the SPL/RGS18/SHP-1 complex by a mechanism distinct from platelet agonists (Ma et al. 2015a).

From a systems perspective, this suggests that crosstalk between platelet activation pathways, the cAMP-dependent regulatory network, and the proteins that sequester RGS18 in resting platelets produces three states with respect to the availability of free RGS18. In resting platelets (State #1), some of the RGS18 is bound to phosphorylated spinophilin in a heterotrimeric complex that includes SHP-1. Some of the remaining RGS18 is bound to 14-3-3γ, in a complex dependent on serine phosphorylation of RGS18. When platelets are activated by thrombin or TxA_2 (State #2), SHP-1 is activated and the SPL/RGS/SHP-1 complex decays. At approximately the same time, RGS18 phosphorylation on Ser49 increases, as does the amount of RGS18 bound to 14-3-3γ (Gegenbauer et al. 2012). In a sense,

spinophilin and 14-3-3γ are working against each other in activated platelets, one releasing and one capturing RGS18, but the net result is still a net increase in free RGS18. Spinophilin can bind to G protein-coupled receptors as well as to RGS proteins, suggesting that it may guide RGS proteins into the vicinity of these receptors (Wang et al. 2004). State #3 occurs when resting platelets are exposed to PGI_2, causing cAMP synthesis to increase. In the presence of cAMP, RGS18 becomes phosphorylated on Ser216 and dissociates from 14-3-3γ (Gegenbauer et al. 2013). At the same time, spinophilin becomes phosphorylated on Ser94, displacing RGS18. The release of the RGS18 from both of its binding partners contributes to the increase in free RGS18 that we observed in PGI_2- and forskolin-stimulated platelets and would be expected to contribute to the well-described resistance of these platelets to activation by platelet agonists (Fig. 4) (Ma et al. 2015a).

Signaling and Structure

Observational studies show that the hemostatic thrombi develop a characteristic heterogeneous structure in which a core of fully activated, densely packed, and stably adherent platelets is overlaid with a shell of less activated, more loosely packed, and less stable platelets (Figs. 1 and 2). When illuminated with antifibrin antibodies or fluorescent fibrinogen, fibrin is found primarily in the thrombus core as well as in nearby tissue planes if substantial leakage of plasma occurs before hemostasis is achieved (Stalker et al. 2013). For technical reasons having to do with the ability to resolve events through vascular walls, most of these studies have been performed in the microcirculation, typically using exteriorized mesentery and cremaster muscle preparations. Conditions in the macrocirculation are considerably different and it remains to be seen whether everything that has been observed in arterioles and venules will map to arteries and veins (Welsh et al. 2015; Cooley 2015; Getz et al. 2015).

Viewed from a systems perspective, the platelets depositing first at a site of vascular injury alter the local environment in which fibrin generation and subsequent platelet activation occur (Fig. 1). Initially, this is because the first platelets to arrive are activated by collagen and thrombin, thereby helping to recruit additional platelets by releasing ADP and TxA_2. Later it is because as platelets accumulate they act as obstacles to the movement of soluble platelet agonists as well as to the escape of red cells and plasma, creating protected zones that can allow local agonist concentrations to rise. Intravital studies with a fluorescent thrombin biosensor show that detectable thrombin activity is confined to the thrombus core region, consistent with the localization of fibrin (Welsh et al. 2012). Computational studies combined with intravital studies using fluorescent albumin and dextran show that intrathrombus transport rates slow even further as platelets within the thrombus core contract, pulling each other into even greater proximity (Fig. 5) (Stalker et al. 2014; Tomaiuolo et al. 2014). Notably, this drop in transport rates precedes the appearance of P-selectin on the platelet surface, a commonly used marker of platelet activation (Welsh et al. 2014). In this section, we will consider how thrombus structural heterogeneity both reflects and alters agonist distribution and, therefore, information flow through the platelet signaling network.

The current map of the platelet signaling network derives in large part from studies in which human platelet responses to agonists were measured ex vivo, either under semistatic conditions produced in test tubes and aggregometer cuvettes or under flow conditions in collagen-coated flow chambers and microfluidic devices. Essential advances have been made by studying humans with rare inherited defects and through studies on genetically engineered mice. Numerous platelet agonists have been identified ex vivo and the most critical ones studied in vivo. The ex vivo studies have identified the key components of the platelet signaling network and defined how platelets respond to individual agonists, but they fall short when it comes to explaining why structural heterogeneity arises. The hemostatic response normally takes place in a setting in which multiple agonists are present and the ability of each to reach critical concentrations is opposed by the continued flow of blood and the presence of inhibitors. When studied ex vivo all of the platelets typically have equal access to whichever agonist is being tested. Within relatively narrow limits revealed by flow cytometry, all of the platelets become activated to a similar extent.

In vivo this is not the case. Collagen exposed by vascular injury is embedded in a connective tissue matrix, incapable of coming into direct contact with more than an initial layer of platelets. As noted earlier, the observed localization of fibrin to the core of the thrombus suggests that thrombin is not present throughout the thrombus, a hypothesis supported by studies with the thrombin biosensor (Welsh et al. 2012). Infusing hirudin to inhibit thrombin reduces the size of both core and shell (Stalker et al. 2013), as does deletion of the mouse platelet thrombin receptor, PAR4 (Vandendries et al. 2007). Very different results are obtained when the role of ADP is examined. Unlike thrombin, there are no biosensors for ADP currently available that can be used in vivo. However, studies performed with the direct-acting $P2Y_{12}$ antagonist, cangrelor, and the $G_{i2\alpha}$(+/G184S) mice show that the thrombus shell is primarily driven by ADP. Infusion of cangrelor reduces the size of the shell without affecting the size of the core, while the RGS10/RGS18-resistant $G_{i2\alpha}$(G184S) substitution increases the size of the shell (Signarvic et al. 2010; Stalker et al. 2013).

It has been known for decades that fibrin clots retract as platelets contract, pulling on fibrin that is bound to the platelet surface via $\alpha_{IIb}\beta_3$ integrin. Outside-in signaling

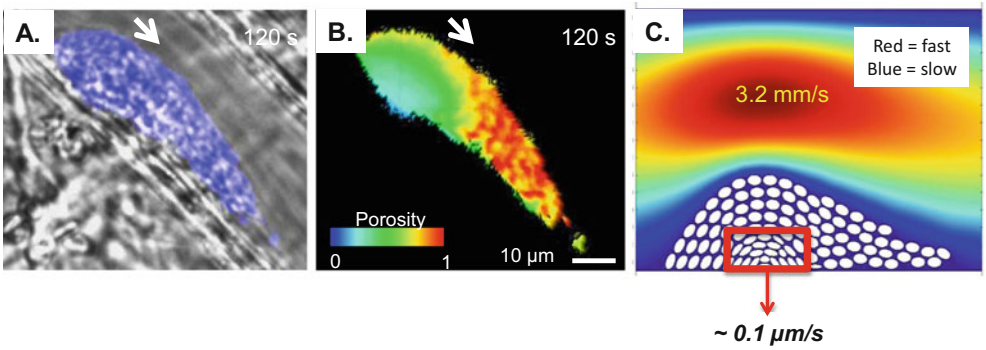

Fig. 5 Packing density and transport rates. Albumin labeled with AlexaFluor 488 was infused into the mouse prior to laser injury in the exteriorized cremaster muscle. Fluorescence was monitored within the lumen and the body of the resultant hemostatic thrombus as it developed. (**a**) A representative image of platelets (*blue*) superimposed over the bright field image captured 120 s after injury. (**b**) A pseudo-colored image of the same thrombus with colors assigned to the labeled albumin chosen to reflect the porosity based on the normalized fluorescence intensity. (**c**) Reconstruction in silico of the core/shell architecture based on experimental data. The smallest gaps between platelets were selected as 10 nm in the core and 200 nm in the shell. The inlet vessel velocity was modeled as a pressure-driven parabolic profile with maximum velocity of 2 mm/s. The computed intraluminal plasma velocity and the average intrathrombus plasma velocity in the core are shown. Platelet packing density, but not the core location, determines the average velocity between platelets (Tomaiuolo et al. 2014). Adapted from Welsh et al. (2014) and Tomaiuolo et al. (2014)

through the integrin is a primary driver for platelet contraction. Platelets bearing a double tyrosine to phenylalanine substitution (diYF) in the cytoplasmic domain of β_3 behave normally in single platelet assays but have a defect in clot retraction assays (Law et al. 1999; Stalker et al. 2014). When observed in vivo thrombi containing diYF platelets have a smaller core and accumulate less fibrin (Stalker et al. 2014). Transport studies show that soluble plasma-borne molecules move more rapidly through hemostatic thrombi formed in diYF mice than in matched controls, suggesting that the smaller core, diminished platelet activation, and reduced fibrin accumulation reflect reduced thrombin retention. Computational studies show that as the gaps between platelets grow narrower, transport becomes diffusion limited rather than convection driven (Fig. 5) (Tomaiuolo et al. 2014). Those studies are consistent with the experimental evidence showing diminished platelet activation and fibrin deposition when platelet contraction is diminished.

Is Any of This Clinically Relevant?

Aside from insights into platelet biology, there are a number of ways that applying a systems biology perspective to hemostatic thrombus formation can inform decision making by cardiologists, hematologists, and pharmaceutical companies. First, it provides a context to better understand why platelets express receptors for so many agonists, not all of which are sufficient to drive full platelet activation in vitro. There is considerable evidence that optimal platelet activation calls for simultaneous signaling through G_q, G_i, and $G_{12/13}$ family members, each of which is responsible for separate signal transduction pathways. This can be accomplished by a single potent agonist, such as thrombin, or by combinations of agonists (Jin and Kunapuli 1998). In vivo, agonists are not distributed evenly. The studies summarized in this chapter suggest that agonist concentration gradients form early in thrombus development. Critical concentrations are reached in different regions within the thrombus, which means that individual platelets within the thrombus are exposed to different combinations of agonists and these combinations can vary over time as well as space. The strengths and limitations of many commonly prescribed antiplatelet agents reflect not only their half-lives, affinities, and off-rates, but also where they work on the platelet signaling network and how well they can penetrate thrombus structure in the setting of existing disease. For example, the results with cangrelor, if generalizable to other $P2Y_{12}$ antagonists, suggest that this family of drugs limits thrombus growth and reduces the risk of recurrent thrombotic events by destabilizing the thrombus shell. This is achieved without undue bleeding risk because in many settings, the remaining thrombus core appears to be sufficient to achieve hemostasis. In contrast, a thrombin inhibitor, such as heparin, will disrupt core formation if administered before thrombus formation is initiated, but may not be able to reach thrombin in the core region of preexisting thrombi.

Second, a systems perspective on the platelet signaling network provides a different context for estimating efficacy and risk, either with new antiplatelet targets or new combinations of antiplatelet agents. Conceptually, it is safer from the hemostasis perspective to target events in the thrombus shell or, perhaps, the core-to-shell transition zone, than it is to target the core, although targeting the

core with thrombin and factor Xa inhibitors such as heparin and apixaban may be most effective in preventing thrombosis.

Third, in an era of personalized medicine, tests of on-treatment platelet function need to be better evaluated to establish their ability to work in vivo. A systems perspective the design of such tests to better reflect the complex conditions within a growing thrombus. Computational studies that recapitulate platelet accumulation and transport, may prove helpful in this regard, especially as the simulations become increasingly refined (Voronov et al. 2013; Tomaiuolo et al. 2014; Flamm et al. 2012; Wang and King 2012; Leiderman and Fogelson 2011, 2013; Kim et al. 2013; Xu et al. 2010).

Fourth, the studies described here help explain why most arterial thrombi arising from vascular disease do not initially become fully occlusive. For example, if thrombus structure in coronary arteries in the setting of atherosclerotic disease mirrors the structures described here, then the instability of the thrombus shell contributes to the failure of thrombus growth as flow rates rise in the partially obstructed arterial lumen. Finally, the perspectives described here provide new potential targets for antiplatelet agents, among them the RGS proteins, RGS10 and RGS18, and their binding partners in resting and activated platelets.

Take Home Messages

1. The platelet activation state is governed by a flexible signaling network that includes repressors as well as enhancers.
2. The enhancers promote platelet activation when vascular injury occurs. The repressors shape the hemostatic response, preventing the local accumulation of excessive numbers of platelets.
3. The elements of the signaling network that are active within each individual platelet at any given time vary not only with the time after injury, but also with the location of the platelet within the growing thrombus or hemostatic plug.
4. Location is a factor because as platelets crowd close to each other, they limit the movement of soluble agonists within the gaps between platelets, creating agonist concentration gradients. The extent of platelet activation is then determined not only by which agonists come into contact with each platelet but also by the local concentration of the agonist.

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Platelet-Derived Microparticles

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Abstract

Numerous findings have allowed the field of microparticles (MP) to progress dramatically from the original discovery of platelet dusts characterizing the phenotypes and functions of these novel subcellular biovectors, investigating the mechanisms of their formation from parental cells, determining their effects on others cells both in physiology and disease states, and translating these notions to disease innovative diagnosis and treatment. The present chapter focuses on platelet-derived MP (PMP) characteristics, biogenesis, and biological roles. After a rapid overview of detection methods, PMP paradoxical functions in disease process and regenerative medicine are discussed, together with future prospects as diagnostic tools and therapeutic agents. PMP are naturally diffusible vectors that play a role in cell/cell communication and deliver active biomolecules. This is believed to have significant pathological implication in the development of several diseases such as cancer, thromboembolism, and atherosclerosis, among others. There is still much research required to understand the paradoxical roles played by PMP in triggering pathological events but also sustaining vessel growth and tissue regeneration. Clinical data linking PMP to pathologies should be interpreted with some caution due to the lack of standardization and technical limitations of methods for PMP exploration that have been applied so far. The recent progress in new methodologies addressing the pitfalls of current techniques in PMP isolation and detection, capable of detecting and counting PMP more accurately, to assess their variability and measure their functional activity, will contribute to a better understanding of their pathological role and also their potential promising use in regenerative medicine.

Introduction

The focus of the emerging field of microparticle (MP) research is centered upon characterizing the phenotypes and functions of these novel subcellular biovectors, investigating the mechanisms of their formation from parental cells, determining their effects on others cells both in physiology and disease states, and translating these notions to disease innovative diagnosis and treatment. In line with platelet biology, while many questions still remain unanswered, numerous findings within the past 40 years have allowed the field of MP to progress dramatically from the original discovery of platelet dust by Peter Wolf in 1967. The present chapter will focus on platelet-derived MP (PMP) characteristics, biogenesis, and biological roles. After a rapid overview of detection methods, PMP paradoxical functions in disease process and regenerative medicine will be discussed, together with future prospects as diagnostic tools and therapeutic agents.

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Characteristics of Platelet-Derived MP

Platelets, like all cells, release different types of extracellular vesicles in response to cell activation and apoptosis including MP (also called microvesicles) and exosomes. While specific characteristics once vesicles are formed are lacking, these distinct entities are classically distinguished one from another based on their size, their content, the mechanisms leading to their formation, and from a practical point of view, from their mode of preparation (Table 1).

The definition of PMP started from seminal experiments conducted by Peter Wolf in 1967 (Wolf 1967) "*to provide evidence for the occurrence in normal plasma, serum and fractions derived from coagulant material of minute particulate form sedimentable by high speed centrifugation and originating from platelets but distinguishable from platelets*." Using ultracentrifugation separation, coagulation tests, and electron microscopy analyses, he observed subcellular coagulant material called "platelet *dust*." Therefore, 40 years ago, Peter Wolf revolutionized previous blood coagulation literature with his discovery of "platelet dust," which was subsequently replaced by the term of PMP.

PMP are cellular membrane fragments with a size ranging from 0.1 to 1 μm that are released in body fluids (Wolf 1967). In addition to plasma and serum, the presence of PMP has been described in vitreous fluid (Chahed et al. 2010); atherosclerotic plaques (Mallat et al. 1999; Leroyer et al. 2007); and in inflammatory fluids, such as bronchoalveolar fluid (Guervilly et al. 2011; Mutschler et al. 2002), synovial fluid (Berckmans et al. 2002), or pleural fluid (Park et al. 2013). Initially identified as an activity supporting thrombin generation in platelet poor plasma, PMP were subsequently characterized on the basis of their surface antigens. As reported for other MP subtypes, PMP externalize phosphatidylserine (PS) and thus bind annexin V in a calcium-dependent manner. They express platelet markers including glycoproteins GPIb and GPIIbIIIa, Platelet-Endothelium Cell Adhesion Molecule (PECAM-1), P-selectin, CD40 ligand (CD40L), and CXC Receptor 4 (CXCR4). However, the surface protein content may be different from that of the plasma membrane of parent cell, since the selective sorting of molecules into PMP can be regulated by the stimuli from specific agonists or microenvironments of the parent cells. When characterized by proteomic and functional studies, PMP derived from activated platelets have been separated into size classes that are heterogeneous and significantly differs in their content by membrane receptors, chemokines, and growth factors (Dean et al. 2009). Platelets carry growth factors, inflammatory mediators and cytokines, lipids, and proteases that are related to their involvement in angiogenesis, inflammation, immune response wound healing, and cell differentiation. PMP also have the ability to scavenge nitric oxide, generate reactive oxygen species, and cleave cellular surfaces proteins via metalloproteinases (Burger et al. 2013).

PMP are the most abundant MP in blood circulation, constituting approximately 70–90 % of circulating MP (Horstman and Ahn 1999; Joop et al. 2001; Berckmans et al. 2001). Studies on the half-life of MP indicate that they are rapidly cleared in the circulation (Flaumenhaft 2006; Rand et al. 2006) suggesting that PMP would need to be constantly shed from platelets from healthy individual to produce the concentration PMP circulating in the plasma of healthy individual. An alternative possibility is that PMP consist of two populations: those derived from platelets following activation and those continuously derived from megakaryocytes, the precursor cells of platelets (Cramer et al. 1997; Flaumenhaft et al. 2009; Cunningham 1995; Wang et al. 2008; Charras et al. 2006; Fackler and Grosse 2008).

In addition to MP, stimulated platelets secrete exosomes, which are smaller vesicles (40–100 nm) released as a consequence of the fusion of intraluminal membrane-bound multivesicular bodies (MVB) with the membrane, in an exocytic manner (Thery et al. 2002). In activated platelets, exosomes are formed by fusion of alpha granules and MVB with the plasma membrane. Besides differences in mechanism of formation, exosome and PMP appear morphologically, phenotypically, and functionally different (Table 1).

Table 1 Characteristics of microparticles and exosomes derived from platelets

	Exosomes	Microparticles
Size	30–100 nm	100–1000 nm
Morphology	Round	Heterogeneous
Markers	MHC, Tetraspanin (CD63, CD9, CD81, CD82, CD53), RabGTPase, Annexins, Alix, Tsg101, HSP	CD42b, CD31, CD41/CD61
		CD62P, PECAM-1, P selectin, CD63, LAMP
PS expression	–	+
Origin	Exocytose of multivesicular bodies	Vesiculation of plasma membrane
Composition	Proteins, mRNA, miRNA	Proteins, mRNA, miRNA
Method	Centrifugation > 100,000 g	Centrifugation < 100,000 g
	Sucrose density gradient: 1.13–1.19 g/ml	

MHC major histocompatibility complex, *PECAM-1* platelet endothelial cell adhesion molecule, *LAMP* lysosome-associated membrane protein, *HSP* heat shock proteins, *Tsg101* tumor susceptibility gene-101, *miRNA* micro RNA, *mRNA* messenger RNA

For example, exosomes contain a very low proportion of PS if any, and have little interaction with factor X, prothrombin, or annexin V, indicating a poor or absence of effectiveness in coagulation reactions (Heijnen et al. 1999). Platelet exosome enrichment in CD63 and to a lesser extent in P-selectin suggests that they may serve specialized functions, supporting heterotypic signaling in the environment of adhering platelets and neutrophils (Heijnen et al. 1999; Denzer et al. 2000); however, none of these markers are specific of a PMP subset.

Biogenesis of PMP

MP formation results from plasma membrane budding, due to remodeling of membrane phospholipids and cleavage of cytoskeleton proteins. This process is a universal feature which occurs virtually in all cell types that are undergoing apoptosis or are being subjected to various types of stimulation or stresses. Under resting conditions, phospholipids are asymmetrically distributed in the plasma membrane, the inner leaflet containing negatively charged phospholipids such as PS and phosphatidylethanolamine. Neutral phospholipids (phosphatidylcholine and sphingomyelin) are enriched on the outer leaflet (Manno et al. 2002; Seigneuret et al. 1984; Daleke 2003). Such asymmetry is maintained by phospholipids transporters. Research over the last few years has revealed important roles for calcium-, mitochondrial-, and caspase-dependent mechanisms leading to PMP formation (Fig. 1). Indeed, in response to platelet activation, PS is exposed at the outer leaflet as a consequence of the calcium-dependent dysregulation of scramblases, floppase/ABC1, and translocase/flippase activities (Daleke 2003; Hamon et al. 2000; Zwaal and Schroit 1997). Recently, scramblase has been identified as anoctamin TMEM-16F (transmembrane protein-16F also called anoctamin 6) (Suzuki et al. 2010; Brunner et al. 2014). Membrane phospholipid remodeling also requires cytoskeleton reorganization involving calcium-activated proteases such as caspases and calpains. The exact role of transmembrane ion transport (Heemskerk et al. 2002; Bucki et al. 2006), transient transmembrane pore, and Store-Operated Calcium Entry (SOCE) in PS exposure deserves further investigations to gain insight into a potential

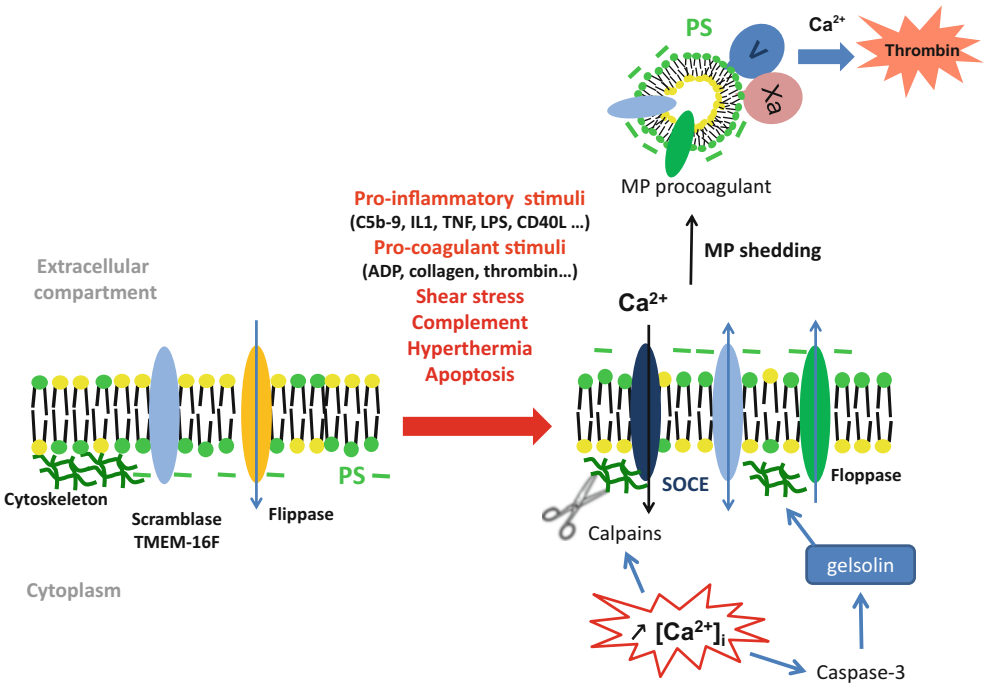

Fig. 1 Biogenesis mechanisms of PMP. At rest (*left figure*), phospholipids in the plasma membrane have an asymmetrical distribution, especially phosphatidylserine negatively charged, located in the inner leaflet. Cytoskeletal reorganization involves proteases activation by cytosolic calcium such as caspase-3 and calpains. Intracellular calcium is needed to increase the exposure of phosphatidylserine and involves different mechanisms: transmembrane pores and store-operated Ca(2+) entry (SOCE). During cell activation or apoptosis, reorganization of the cytoskeleton is dependent on the activation of rho-associated coiled-coil-containing protein kinase 1 (ROCK-I), ultimately resulting in the release of MPs into the extracellular fluid. The rearrangement of the cytoskeleton leads to budding of the membrane and release of MP that expose PS, membrane antigens, and cytoplasm components. The presence of PS on the outer leaflet of MP provides a catalytic surface able to assemble coagulation enzymatic complexes and to generate thrombin. *Ag* antigen, *PS* phosphatidylserine, *TMEM-16F* transmembrane protein-16F, *SOCE* store-operated calcium entry, *LPS* lipopolysaccharide, *TNF* tumor necrosis factor, *ADP* adenosine diphosphate, *IL1* interleukin 1, Ca^{2+} calcium, *MLC* myosine light chain

pharmacological control of PMP shedding. More recent studies indicate that mitochondrial membrane depolarization is an integral event leading to PS externalization (Lopez et al. 2008; Leytin et al. 2009). Taken together, the phospholipid transient mass imbalance between the two leaflets and the proteolysis of the cytoskeleton triggered by calcium-activated calpains induces the shedding of PMP. Compared to other cells, platelets have the highest scrambling rate known, which ensure a rapid and localized blood coagulation. It is important to emphasize that rapid phospholipid membrane remodeling and PS exposure characterize a physiological relevant procoagulant response. Accordingly, PS borne by MP provides accessible sites for the assembly of the coagulation complexes, leading to thrombin generation and clotting. In this context, shed PMP could protect from bleeding during idiopathic thrombocytopenic purpura (Jy et al. 1992). The physiological relevance of platelet membrane remodeling and PMP shedding is also well exemplified in Scott syndrome. This rare bleeding disorder is characterized by provoked hemorrhages caused by reduced floppase activity of cells, diminished externalization of PS, and reduced MP shedding (Toti et al. 1996). Several authors have described mutations in the gene encoding TMEM16F in these patients (Castoldi et al. 2011; Suzuki et al. 2010).

PMP shedding occurs after physiological activation of platelets by thrombin, adenosine diphosphate or collagen (Sims et al. 1989; Wiedmer et al. 1990; Italiano et al. 2010; Zwaal et al. 1992) or other agonists or stimulating conditions including calcium ionophore, CD40L, and oxidative stress (Morel et al. 2004; Simak and Gelderman 2006; Horstman et al. 2004). PMP shedding has also been reported following exposure of platelets to purified complement components (Sims et al. 1988). One hypothesis is that recovery of membrane potential may follow shedding of PMP carrying the C5b-9 pore membrane attack complex (MAC), on the basis that PMP from platelets exposed to MAC carried the majority of available MAC. Such hypothesis is substantiated by the observation of increased levels of PMP in Paroxysmal Nocturnal Hemoglobinuria, a pathological setting where complement-mediated platelet activation occurs (Hugel et al. 1999). It has been reported that high shear forces trigger platelet activation and PMP release. Activation of protein kinase C promotes shear-dependent PMP formation (Miyazaki et al. 1996; Holme et al. 1997). When high shear forces are applied in the microcirculation, thrombin generated in the vicinity of the hemostatic plug is crucial for PMP generation, with a correlation between platelet-activating factor release and PMP biogenesis (Iwamoto et al. 1996; Chow et al. 2000). The capacity of MP to be generated by physical reactions occurring during blood product manipulations, such as shear forces, contact surfaces, and senescence, makes PMP an integral part of transfusion medicine. Platelet activation induced by antibodies can also be accompanied by the formation of PMP. Immunoglobulins (Ig)G to heparin-platelet factor four complexes as observed in heparin-induced thrombocytopenia (HIT) generate PMP (Untch et al. 2002). This property is the basis of a PMP generation assay for HIT diagnosis (Mullier et al. 2014). Similarly, autoimmune antibodies against platelet antigens are associated with PMP generation. The role of these antibodies is supported by animal models of immune thrombocytopenia where the injection of antiplatelet antibodies in mice induced thrombocytopenia associated with increased circulating PMP (Piguet and Vesin 2002).

Vesicle shedding also occurs during senescence and apoptosis of either anucleated cells like platelets (Zwaal et al. 1992) and nucleated cells like the megakaryocytes (Flaumenhaft et al. 2009). Before cells undergo nuclear apoptotic DNA fragmentation, they show early cell membrane asymmetry and expose PS (Yang et al. 2002). Platelets contain several key regulators of apoptotic cell death including caspases, different members of the B-cell lymphoma (Bcl)-2 protein family, and mitochondrial pathways (Shcherbina and Remold-O'Donnell 1999; Zhang et al. 2007; Jobe et al. 2008). It was demonstrated that the platelet apoptotic machinery and the classical calcium-derived rapid agonist pathways, although both able to promote PS exposure, are distinct. For example, hyperthermia has been identified as a physical condition known to affect PMP biogenesis by triggering apoptotic events, including caspase 3-dependent gelsolin cleavage and PS exposure (Wang et al. 2010). The coexistence of different signaling pathways triggered either by activation or apoptotic stimuli is not restricted to platelets. It has also been reported for endothelial cells and reflects distinct mechanisms leading to the release of extracellular vesicles (Dignat-George and Boulanger 2011).

Biological Functions of PMP

PMP have crucial roles in different biological functions: hemostasis, host defense, response to injury, and immune response (Fig. 2).

The procoagulant properties of MP rely mainly on the expression of anionic phospholipids, especially PS which provide a catalytic phospholipid surface for the assembly of blood coagulation factors: FII, VII, IX, and X, as well as the assembly of the coagulation complexes, accelerating the formation of thrombin (Morel et al. 2006). Interestingly, although this phospholipid-dependent procoagulant activity appears to be a common mechanism between the different MP subsets, PMP membrane is among the most efficient to contribute to the thrombin generation and fibrin formation. PMP isolated from major thalassemia patients are more procoagulant than MP derived from erythrocyte. This

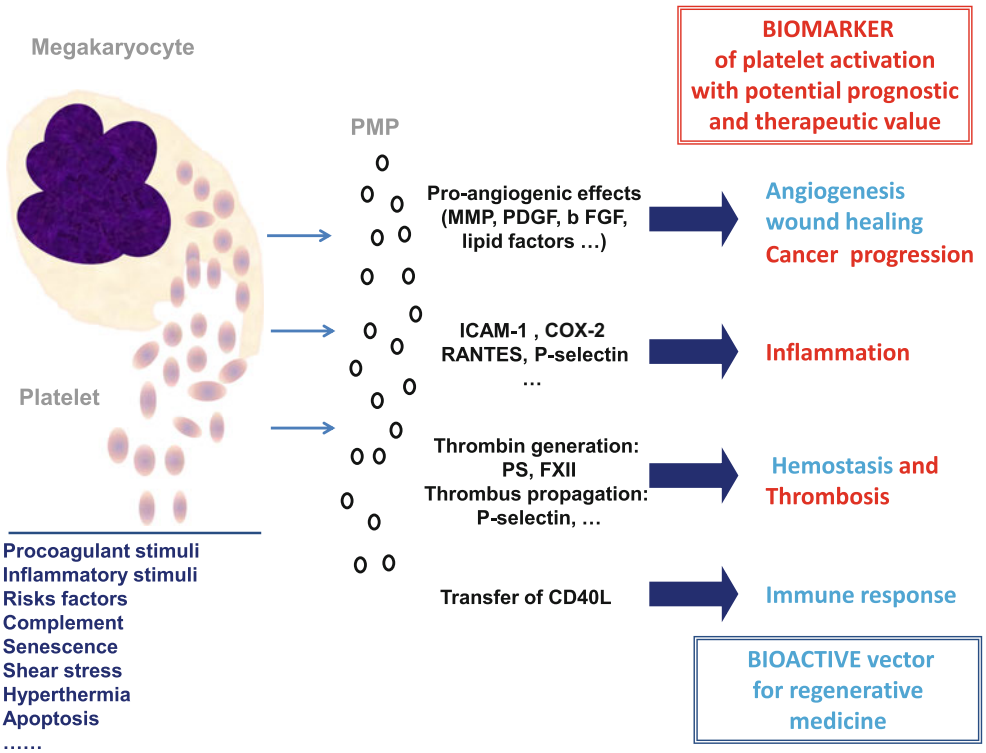

Fig. 2 Paradoxical biological functions of PMP. PMP might have deleterious effect (shown in *red*), such as promoting cancer progression or stimulating inflammation and thrombosis. Alternatively, PMP might have beneficial effect (shown in *blue*) because of angiogenesis and wound healing process and in immune response by collaborating to B-cells. *PDGF* platelet-derived growth factor, *b FGF* basic fibroblast growth factor, *ICAM-1* intercellular adhesion molecule 1, *COX-2* cyclooxygenase 2, *PS* phosphatidylserine, *VEGF* vascular endothelial growth factor, *FGF-2* fibroblast growth factor 2, *RANTES* regulated on activation, normal T expressed and secreted

activity is further amplified after splenectomy independently of the PMP numbers (Agouti et al. 2015). PMP were also suggested to express tissue factor (TF) (Schwertz et al. 2006); however, this expression is controversial and may result in part from the fusion of monocyte-derived TF^+ (positive) MP with activated platelets. Because TF-dependent procoagulant activity of PMP seems limited (Owens and Mackman 2011), it is unlikely that PMP can provide a major contribution to the pool of TF^+ MP present in healthy individuals and in patients (Lacroix et al. 2013a). However, as recently described, platelet and erythrocyte MP not only propagate coagulation by exposing PS but also initiate thrombin generation independently of TF in a factor XII-dependent manner (Van Der Meijden et al. 2012). Interestingly, PMP vectorize the plasminogen activator inhibitor (PAI)-1 and contrary to endothelium-, leukocyte-, or tumor-derived MP, they are devoid of fibrinolytic activity which may counterbalance their procoagulant activity (Lacroix et al. 2012b; Lacroix and Dignat-George 2013).

Many studies have described the involvement of circulating PMP in inflammation. In fact, PMP constitute an important source of substrate for phospholipase A2, generating lysophosphatidic acid, a potent proinflammatory mediator and platelet agonist (Barry et al. 1999). Arachidonic acid, transported by MP, is able to stimulate the expression of cyclo-oxygenase-2 on endothelial cells and monocytes and thereby the production of prostaglandins (Barry et al. 1997). The arachidonic acid transfer also induces membrane expression of ICAM-1 (intercellular adhesion molecule 1) on the endothelium which will consequently stimulate monocyte adhesion to the surface of endothelial cells (Barry et al. 1998). They may also facilitate the recruitment of many immune cells (monocytes; neutrophils; lymphocytes T, B; and Natural Killer cells), in particular, by the interaction between P-selectin on PMP and P-selectin glycoprotein ligand-1 on neutrophils (Ogura et al. 2001; Forlow et al. 2000; Baj-Krzyworzeka et al. 2002). Platelets can interact directly with the activated endothelium by increasing the monocyte recruitment after the transfer by PMP of chemokines such as RANTES (Regulated on Activation, Normal T Expressed and Secreted) (Mause et al. 2005).

Platelets play also an important role in modulating immunity (Semple and Freedman 2010). While there is support for the concept that PMP release play a role in this regulation, the underlying mechanisms by which they provide early signals to immune cells are not fully understood. Sprague et al. have shown that PMP activate adaptive immune cells in specific tissue compartments in a CD154-dependent manner that trigger antibody synthesis and alter lymphocyte activities (Sprague et al. 2008). Using an adoptive transfer model, the authors show that PMP transfer activation signals to B-cells. PMP transport and deliver CD154 (CD40L) to turn on antigen-specific immunoglobulin G production and germinal center formation. The release of PMP from sites of thrombosis in disease states may, therefore, stimulate an immune response.

Several studies have shown that PMP have the capacity to induce angiogenesis and are involved in the metastasis of cancer. Indeed, PMP promoted the proliferation, survival,

migration, and tube formation of human umbilical vein endothelial cells, suggesting that PMP may favor the formation of new blood vessels during tumor growth. This effect was mediated by the concerted action of fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and lipid factor such as sphingosine 1-phosphate (S1P), lysophosphatidic acid, and phosphatidate (Kim et al. 2004). PMP also induced sprouting of blood vessels both in vitro and in vivo (Brill et al. 2005). Furthermore, intramyocardial injection of PMP markedly elevated the amount of new capillaries formed in the heart muscle in the background of ischemia (Horstman et al. 2007).

PMP play also a significant role as vector in the transcellular communication as they possess various types of receptors and bioactive substances on their surface including cytokines, signal proteins, and nucleic acids, in addition to mRNA and mi-(micro)RNA (Wu et al. 2013; Freyssinet 2003). They exchange biological information with target cells such as, for example, monocytes or endothelial cells either via surface-expressed ligands or by transporting surface receptors from one cell to the other. MP also has the capacity to reprogram target cells via attaching or fusing and delivering cytoplasmic proteins and RNA to the cells. The fusion of PMP membranes to target cell membranes was demonstrated to transfer the surface protein CXCR4 to cells causing recipient cells (glioblastoma and hematopoietic cell lines or erythroblasts) susceptibility to human immunodeficiency virus infection (Rozmyslowicz et al. 2003). Arachidonic acid is a lipid mediator delivered by PMP that can be further processed into thromboxane A2 by recipient platelets or endothelial cells and contribute to their activation (Barry et al. 1997). Several works showed that PMP contain transcription factors, such as peroxisome proliferator-activated receptor (PPAR)γ (Ray et al. 2008), RuvB-like 2, Signal Transducer and Activator of Transcription (STAT)3, and STAT5A (Garcia et al. 2005) derived from platelets. Transcription factors are transported from platelets through MP and retain function within monocytes (Ray et al. 2008). Human platelets activated by thrombin release their miR-223 contents in PMP that can be internalized by Human umbilical vein endothelial cells (HUVEC). Functional Argonaute 2 (Ago2)/miR-223 complexes present in PMP were shown to regulate the expression of a reporter gene in recipient HUVEC, exerting heterotypic regulation of gene expression of endothelial cells (Laffont et al. 2013). Thus, cocultures of PMP with various cell types further allow to investigate their role in the intercellular communication.

Detection Methods and Assays for MP Determination

Platelets are easily activated in vitro; therefore, accurate characterization of PMP requires very rigorous preanalytical handling and processing of blood samples (i.e., phlebotomy, transport, plasma preparation, and storage). Controlling these steps is a prerequisite to avoid artifactual generation of PMP (Ayers et al. 2011; Lacroix et al. 2012a; Artoni et al. 2012), to allow intra- and interlaboratory standardization and data interpretation (Lacroix et al. 2013b). In the field of the International Society of Thrombosis and Haemostasis (ISTH), recommended protocol for isolation of PMPs from whole blood samples has been proposed for MP assessing in clinical samples. Important experimental factors to consider include (1) using a large needle size (21G minimum), (2) discarding the first milliliters of blood, (3) using appropriate anticoagulant (citrate) to limit artifactual vesiculation, (4) limiting agitation and horizontal transportation of tubes, (5) delaying by a 2 h maximum the first centrifugation after blood collection, and (6) carefully controlling the centrifugation process in order to remove all platelets before freezing.

Different methodologies have been adapted to characterize PMP (Table 2).

Table 2 Methods of PMP characterization (adapted from *Bartenevaetal et al. BMC Cell Biol. 2013*)

Method	Quantification	Phenotype	Sizes	Limits
Flow cytometry	+	+	Limited	Aggregates, diameter > 200 nm
DLS or NTA	+	–	+	Artifacts (cellular debris and proteins)
RPS	+	–	+	Artifacts (cellular debris and proteins, lipids)
Electron microscopy	Limited	Limited[a]	+	Artifacts during sample preparation
AFM	Limited	Limited[b]	+	Artifacts (cellular debris and proteins)
ELISA	+	+	–	Accessibility of PS or activated molecule carried by MP
Functional tests	+	+[c]	–	Accessibility of PS or activated molecule carried by MP
Mass spectrometry	–	+ (proteins extraction)	–	Sample > 10 μg

AFM atomic force microscopy, *DLS* dynamic light scattering, *ELISA* enzyme Linked ImmunoSorbent Assay, *NTA* nanoparticle tracking analysis, *RPS* resistive pulse sensing

[a]Annexin V and specific monoclonal antibodies labeled with gold beads of different diameters (up to 3 simultaneous markings)

[b]Development of surfaces coated with specific monoclonal antibodies

[c]By capture on magnetic beads

Among physical methods, flow cytometry (FCM) remains the most commonly used technique with the highest potential to determine the MP origin (van der Pol et al. 2014). Over the past few years, significant improvements in the sensitivity to detect vesicles of smaller size have confirmed this methodology as the most promising for routine enumeration of MP subsets (Robert et al. 2012; Hoen et al. 2011; Zhu et al. 2014). The possibility of simultaneously detecting two or more antigens on MPs by antibodies conjugated with different chromophores is the main advantage of the method. Generally, annexin-V, which has a high affinity for PS, is used as a general marker for MP despite the fact that not all MP expose PS. Its combination with a platelet marker such as integrin-α_{IIb} (CD41), which is the most specific, CD-61, CD42b, or P-selectin (CD-62P) allows to identify PMP. This method is also the first to benefit from standardization efforts coordinated by the ISTH standardization committee. Thus, several dedicate tools have been developed such as Megamix beads and help to improve the intra and interinstrument reproducibility (Lacroix et al. 2009, 2010; Poncelet et al. 2015). Combination of this methodology with impedance or fluorescence microscopy opens interesting perspective that needs further developments before significantly adding to the methodological toolbox of PMP assessment (Zwicker 2008; Zwicker et al. 2012; Headland et al. 2014).

To overcome the lack of sensitivity of FCM to detect MP of smaller size, alternative methodologies based on light scattering or impedance are available. Dynamic light scattering (DLS) analyzes PMP exposed to monochromatic light from a laser. PMP scatter the light and produce Brownian motion that can be analyzed to determine the size distribution (Lawrie et al. 2009). Although, determination of the PMP concentration of platelet-rich plasma and apheresis platelet concentrates by DLS was reported (Xu et al. 2011), this method is only accurate in a monodisperse system. Thus, methodologies such as Nanoparticle Tracking Analysis (NTA) allowing to measure the Brownian motion under a laser beam of individual particles have been applied (Dragovic et al. 2011). The light scattered from the particles can be visualized by microscopy, and the particle movements of each MP recorded with a camera allowing to count and measure each MP individually. NTA allowed to detect particles smaller than 100 nm but with a low resolution (van der Pol et al. 2014). The main limitation of this methodology is clearly the lack of specificity of the detected events which may be partially overcome using operation in fluorescence mode through identification of membrane markers. Optimal concentration for counting is in the range of 10^8 to 10^9 MP/ml. Thus, larger PMP are not visible because of the low volume analyzed. Alternatively, Tunable Resistive Pulse Sensing (TRPS) is a technique based on impedance to monitor individual MP of 80–1000 nm or more, as they move through tunable nanopores in elastomeric membranes (Coumans et al. 2014). Nanoparticles are measured due to a change in the electric resistance when they pass through the pores, enabling a determination of the size distribution, surface charge, concentration, and zeta potential of the nano/microparticles present in a complex and polydispersed particle samples. Although this methodology detects events smaller than FMC, it is not possible to distinguish MP from lipoproteins or protein aggregates or to determine the origin subsets of MP.

Microscopy methods have the best sensitivity to visualize PMP and determine their structure but cannot be used as a routine methodology because of the time-consuming process. Among them cryo-transmission electron microscopy which allows to image MP in their native form revealed key information about their diversity in biological fluids (Arraud et al. 2014). Origin of MP can be determined by immunogold labeling. Atomic force microscopy (AFM) allows a very sensitive detection of PMP when CD41-positive PMP are captured by anti-CD41 antibodies bound onto a flat muscovite mica surface, a nonconducting layered mineral. AFM can determine the size, antigenic properties, and numbers of defined subsets of MPs (Yuana et al. 2010).

An other group of method is composed of bulk assays providing a global information from each sample. This include immunoassays, functional assays, and hybrid assays associating capture and function testing determining the procoagulant activity, in relation to the presence of PPL or exposure to PS, TF, or other PMP surface markers. An ELISA method based on the binding of PMP by two monoclonal antibodies against glycoprotein (GP) CD42a (GPIX) and CD42b (GPIb) markers was developed in Japan (Osumi et al. 2001). This assay was used in a multicentric assessment of samples from patients with atherothrombosis and found to yield data similar to FCM (Nomura et al. 2009). Functional assays for MP are mainly based on PS- or TF-dependent procoagulant activity but only tests related to PS are related to PMP. PMP can be quantified by measuring the concentration of negatively charged PPL that induces prothrombinase activity. STA-Procoagulant-PPL* (Stago, Asnières-sur-Seine, France) is a clotting assay to detect procoagulant phospholipids (PPL) based on the time needed to generate a clot when a test sample containing PMP is added to plasma depleted of procoagulant PPL. Coagulation is triggered by the addition of activated factor X (FXa) (Gonzalez et al. 2009). MP-dependent thrombin generation tests can be performed in microplate format using the Calibrated Automated Thrombogram* system (Thrombinoscope BV, Maastricht, NL and Stago, Asnieres, F) (Hemker et al. 2002). A reagent containing TF and a minimal amount of PPL is added to the plasma sample to induce thrombin generation through TF-bearing MP. Thrombin is quantified using a

thrombin fluorogenic substrate and calcium. Finally, hybrid assays such as Zymuphen-MP* technology, commercialized by Hyphen Biomed (Andresy, F) combine solid-phase capture of MP on annexin V or antibody and determination of prothrombinase activity (Jy et al. 2004).

In addition, proteomic approaches are very useful for determining the protein composition of PMP and for elucidating their physiological functions. PMP contents have begun to be unveiled using newly developed proteomic approaches. Such exploration involves critical steps to purify PMP. Various analytical methods such as 2D electrophoresis, ultraperformance liquid chromatography, nano high-performance liquid chromatography (HPLC), matrix-assisted laser desorption ionization (MALDI) tandem mass spectrometry, spectral count analysis, etc., can be used for identification (Watts et al. 2012; Capriotti et al. 2013).

Depending on the purpose of PMP characterization (molecular composition, number, size, functional activity, etc.) and the sample nature (plasma, cell supernatant, etc.) an appropriate combination of methods should be made taking into account their current limitations.

PMP in Diseases

PMP could play a critical role in numerous physiopathology conditions: immune and inflammatory disorders, cardiovascular disorders, cancer, and infectious diseases. Boilard et al. have shown the important role played by PMP in inflammatory responses of auto-immune diseases like rheumatoid arthritis by promoting the release of inflammatory interleukines (Boilard et al. 2010) and by the presence of immune complexes on their surface (Cloutier et al. 2013). Platelets accumulate in the joints of rheumatoid arthritis patients and elevated numbers of PMP are found in their synovial fluid. PMP induce an innate immune response via the elicitation of cytokine responses (IL-1) from synovial fibroblasts and the presence of mitochondrial components inside PMP. These reports put forward that PMP might be the culprits involved in the inflammatory and thromboembolic processes in rheumatoid arthritis patients (Knijff-Dutmer et al. 2002).

Because PMP express procoagulant PS on their surface, they may be involved in the pathogenesis of arterial thrombotic and thromboembolic complications (Italiano et al. 2010; Puddu et al. 2010). Acute coronary syndrome is associated with platelet activation and the released PMP which provide a new prothrombotic interface for fibrin, blood cell, and a growing thrombus resulting in narrowing of the vessel. In fact, the PMP contain receptors ($\alpha_{IIb}\beta_3$, Ib, Ia, and IIa) for platelet–subendothelium attachment, and P-selectin for platelet–leukocyte interactions and inflammatory response (Li and Cong 2009). Several studies have evidenced that circulating MP might serve as potential prognostic markers for atherosclerotic vascular disease. PMP that express P-selectin and CD63 on their surface are a sign of platelet activation in peripheral arterial disease and myocardial infarction (van der Zee et al. 2006). PMP contribute also to cardiovascular risk in diabetes mellitus patients. Further, these patients are reported with platelet hyper-reactivity and increased platelet activation due to a multitude of factors (hypersecretion of insulin, hyperglycemia, hyperlipidemia, oxidative stress, endothelial dysfunction, and inflammatory condition). Cohen et al. reported that there was conjecture that the increased thrombin activity, PMP formation, and caspase activity together in the type 2 diabetic blood may contribute to the hypercoagulability of diabetic blood. The study highlighted the link between diabetic platelets and coagulation proteins that lead to prothrombotic condition and validates the increased risk of thromboembolic events in diabetic population (Cohen et al. 2002). PMP were also reported to promote the expression of adhesion molecules in monocytes and endothelial cells and therefore, it appears that they might even participate in the development of atherosclerosis in diabetics (Nomura et al. 2011).

High PMP levels could play a role in cancer, in particular, in tumor progression and reduced response to treatment (Helley et al. 2009). PMP are reported to promote angiogenesis during tumor growth via VEGF, FGF-2, and lipid factor (Brill et al. 2005). In gastric cancer, PMP levels are better forecasters of metastasis than IL-6, RANTES, and VEGF (Kim et al. 2003). PMP can also trigger the secretion of matrix metalloproteinase-2 in prostate cancer cells, promoting tumor invasiveness (Dashevsky et al. 2009).

Activated platelets and MP are reported to play a role in infectious diseases. In particular, in malaria infection in altering the blood–brain barrier leading to severe form of malaria called cerebral malaria (Combes et al. 2006) and in hyperthermia and dengue. In a recent study, it was reported that in the course of dengue infection activated and apoptotic platelets aggregate with monocytes and trigger specific cytokines (IL-1b, IL-8, IL-10, and monocyte chemoattractant protein-1 (MCP-1)) (Hottz et al. 2014).

Although accumulating evidence argues in favor of a key role of PMP in different pathophysiological mechanisms, their specific contribution compared to platelets is not fully established. Animal models where the vesiculation process would be blocked are critically needed to clearly identify PMP as a pharmacological target.

PMP in Regenerative Medicine

Due to their content of bioactive substances entrapped within their granules, it is well established that platelets play a major role in tissue repair, vascular remodeling, and tissue regeneration (Nurden et al. 2008). Platelet alpha

granules contain a large spectrum of growth factors orchestrating tissue regeneration, such as platelet-derived growth factor (PDGF), tumor growth factor (TGF)-beta1, VEGF, epithelial growth factor (EGF), and Brain-derived neurotrophic factor (BDNF). Platelet growth factor preparations are increasingly used in clinics for the healing of soft and hard tissues (Burnouf et al. 2013; Tzeng et al. 2013; Chen et al. 2010). However, little is known about the capacity of PMP *per se* to play a role in tissue repair. Because PMP are implicated in development, angiogenesis, wound healing and more generally, in tissue remodeling, one can hypothesize that they are acting as naturally occurring liposome-like structures, able to have beneficial role for vascular regeneration. The constant presence of PMP in the blood stream has a physiologically significant contribution to maintain the function of healthy vessels (Siljander 2011). The addition of PMP to endothelial progenitor cells enhanced their regenerative capacities in vivo by stimulating their recruitment, migration, and differentiation (Mause and Weber 2010). PMP were also reported to act by inducing the transformation of peripheral blood monocytes into endothelial progenitors, with possible implications for cardiovascular disorders (Prokopi et al. 2009). PMP also plays a role in repair of myocardial muscle as intramyocardial injections induce a marked increase in the amount of capillaries following ischemia (Italiano et al. 2010). Injection of endothelial progenitor cells preconditioned by PMP has also been reported to enhance the effect of therapeutic angiogenesis for limb ischemia, in atherosclerotic patients (Ohtsuka et al. 2013). Local application of PMP, that contain cytokines and growth factors that influence angiogenesis, may emerge as a novel therapeutic strategy for targeting angiogenesis-related conditions (Varon and Shai 2009).

The regenerative role of PMP on hard tissues is suggested by observations showing that these vesicles accelerate bone regeneration in grafting applications, as evidenced by increased mitogenic responses of trabecular bone-derived cells, thereby contributing to regeneration of mineralized tissues (Gruber et al. 2002). PMP were also reported to promote neuronal cell proliferation, survival, and differentiation, suggesting a role in treating brain injuries (Hayon et al. 2012b). Indeed, PMP were found to (1) be internalized in human brain endothelial cells in vitro and modify their function (Faille et al. 2009, 2012); (2) promote angiogenesis and reperfusion in postischemic revascularization in a rat model (Burnouf et al. 2013); (3) increase in vitro survival, proliferation, and differentiation of neural cells (Hayon et al. 2012b; Brill et al. 2005; Mack et al. 2000). The phospho-AKT (protein kinase B) and pERK (extracellular signal-regulated kinase) pathways have proposed as potential mechanisms. Interestingly, these effects were confirmed using in vivo models of cerebral ischemia, where PMP were topically applied to injured brain using a biodegradable polymer (Hayon et al. 2012a; Shan et al. 2013), nicely demonstrating the therapeutic potential of PMP in strokes and possibly in neurological diseases.

Future Directions

In this chapter, we have attempted to highlight that PMP are not merely "platelet dust" exposing procoagulant/prothrombotic molecules but are increasingly believed to act, as naturally diffusible vectors that play a role in cell/cell communication and delivery of active biomolecules. Such cell/cell communication is believed to have significant pathological implication in the development of several diseases such as cancer, thromboembolism, and atherosclerosis, among others. There is still much research required to understand the paradoxical roles played by PMP in triggering pathological events but also sustaining vessel growth and tissue regeneration. The mechanisms involved in the generation of excessive PMP and their association with various pathologies are just beginning to be unveiled. It is a known fact that platelets produce different types of MP in response to different stimuli under varying physiological and pathological conditions. These PMP vary in their composition and thereby in their effect produced on the target cells as well. Therefore, with the background of recent research activities on personalized medicines, the future of PMP would be their application as emergent biomarkers for disease management. Another side would be to generate tailor-made PMP, generated by incorporating specific effectors molecules as well as surface antigens, which target a specific set of cells or tissues. Clinical data linking PMP to pathologies should be interpreted with some caution due to the lack of standardization and technical limitations of methods for PMP exploration that have been applied so far. The recent progress in new methodologies addressing the pitfalls of current techniques in PMP isolation and detection, capable of detecting and counting PMP more accurately, to assess their variability and measure their functional activity, will contribute to a better understanding of their pathological roles and also their potential promising use in regenerative medicine.

Take Home Messages

- Platelet-derived microparticles (PMPs) are not merely "platelet dust" exposing procoagulant molecules but act as naturally diffusible vectors that play a role in cell to cell communication.
- Much research is required to understand the paradoxical roles played by PMP in triggering

(continued)

pathological events but also sustaining vessel growth and tissue regeneration.
- Early clinical data linking PMP to pathologies should be interpreted with due to the lack of standardization and technical limitations of methods.
- The recent progress in PMP isolation and detection will contribute to a better understanding of their pathological role and their potential promising use in regenerative medicine.

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Platelet Interactions with the Blood Vessel Wall

Vascular Control of Platelets

Bradley A. Maron and Joseph Loscalzo

Abstract

Appropriate hemostasis hinges on normal vascular control of platelet function to suppress thrombosis in the absence of blood vessel injury. In this regard, normal intercellular communication between vascular endothelial cells and platelets is integral to maintaining platelets in an inactivated state and inhibiting maladaptive clot (thrombus) formation. Conversely, disruption to endogenous synthesis of nitric oxide, prostaglandin I_2, and endothelial cell surface-associated ecto-ADPase/CD39 occurring due to genetic or acquired factors is an important contributor to the pathogenesis of clinically important thrombotic pathophenotypes, including myocardial infarction and stroke. By analyzing differences in vascular control of platelets according to circulatory bed, particularly with respect to the pulmonary and systemic circuits, understanding the relevance of hypoxia, inflammation, blood flow, and oxidant stress to platelet aggregation and adhesion is enhanced. It is also anticipated that clarifying the biophysical and molecular sequence of events underpinning endothelial regulation of platelet activation will require next-generation methods that utilize whole organ platforms in silico to identify novel mediators of thrombosis in vivo and prevent the attendant adverse consequences in patients.

Abbreviations

AC	Adenylyl cyclase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CTEPH	Chronic thromboembolic pulmonary hypertension
Da	Dalton
H_2O_2	Hydrogen peroxide
IL	Interleukin
IP3	Inositol triphosphate
MLC	Myosin light chain
MLCK	Myosin light chain kinase
$NO^{\cdot}$	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NOx	Nitrogen oxides
NOX	NADPH oxidase
$O_2^{-\cdot}$	Superoxide anion
$ONOO^-$	Peroxynitrite
PAH	Pulmonary arterial hypertension
PAI-1	Plasminogen activator inhibitor-1
PG	Prostaglandin
PKG	Protein kinase G

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ROS	Reactive oxygen species
TXA	Thromboxane A_2
VWF	Von Willebrand factor

Introduction

The endothelium is a thin cell layer lining the blood vessel wall interior that performs various critical functions to maintain vascular hemostasis. While the endothelium operates as a physical barrier to prevent transvascular fluid flux (edema) and extravasation of blood to the extravascular space (hemorrhage), it also regulates vascular function by serving as a cellular source of key effectors that interact with circulating blood cells (Rondina et al. 2013). In this regard, a principal function of vascular endothelial cells is to interface with platelets to inhibit and coordinate thrombosis and hemostasis, respectively, under physiological and pathological conditions, respectively. Thus, although platelets are the central cell type involved in thrombosis and many clinically important blood dyscrasias, mammalian vascular hemostasis must be considered in the context of the platelet–endothelial cell interaction to understand the full scale of hemostasis biology.

Intercellular communication between the platelet and endothelium is driven by dynamic alterations to the protein expression profile of each cell type. This may involve changes in expression of cell-specific ligand(s) or receptor (s), such as upregulation of the integrin CD40L that interacts with $\alpha_{IIb}\beta_3$ in activated platelets to stabilize clot formation (Fuentes et al. 2013). In addition, platelet-derived paracrine factors, such as serotonin, exert control over platelet–endothelial interaction kinetics to increase vascular tone, levels of procoagulant proteins, and thrombus/fibrin synthesis (Weyrich and Zimmerman 2013). Direct transfer of RNA from platelets to endothelial cells is yet an additional mechanism leading to alterations in the programmed response to vascular stress or injury (Risitano et al. 2012; Rondina et al. 2013).

End-clinical pathophenotypes characterized by platelet overactivation, such as myocardial infarction and stroke are due, in part, to failed suppression of platelet activation by the endothelium. Broader pathogenic processes occurring concomitantly with or promoting vascular disease, such as inflammation or atherosclerosis, often trigger inhibition of mechanisms that protect the endothelium from inappropriate thrombosis. Predicting the functional consequences of maladaptive changes to bidirectional signaling between platelets and endothelial cells, however, hinges on blood vessel caliber, anatomic orientation (e.g., systemic vs. pulmonary circulation), and the presence of genetic predispositions to thrombosis.

Endothelial nitric oxide ($NO^{\cdot}$), prostaglandin I_2 (PGI_2), and endothelial cell surface-expressed ecto-ADPase/CD39 are three important bioactive systems that suppress platelet aggregation and adhesion. This chapter will review traditional and novel concepts underlying the molecular basis of platelet–endothelial interactions in the physiological and diseased state with emphasis on these three systems. We will also analyze differences in vascular control of platelet function that are observed between the systemic and pulmonary circulations, and discuss novel in silico technological platforms that aim to clarify the physical and biomechanical underpinnings of the platelet–endothelial interface.

Nitric Oxide

A 30 Da lipophilic gaseous molecule, $NO^{\cdot}$ is synthesized in mammalian tissue via activation of three nitric oxide synthase (NOS) isoforms: endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). These NOS isoforms are homodimeric enzymes containing a calmodulin-binding domain that separates an N-terminal heme-binding domain and a C-terminal reductase domain, and require five cofactors to catalyze the synthesis of $NO^{\cdot}$ from L-arginine (Porter et al. 1990; Maron and Michel 2012). Alternatively, the protonation of nitrite (NO_2^-) to HNO_2 under highly acidic conditions may also result in the spontaneous generation of $NO^{\cdot}$ through the following reactions: $2HNO_2 \rightarrow N_2O_3 + H_2O$, $N_2O_3 \rightarrow NO^{\cdot} + NO_2$ (Maron et al. 2013; Lundberg et al. 2011). Nitric oxide is critical for maintaining an antithrombotic phenotype through its inhibition of platelet activation. In particular, $NO^{\cdot}$ mediates this effect by decreasing bioavailable levels of calcium (Ca^{2+}), inhibiting the thromboxane A_2 (TXA_2) receptor and formation of eicosanoids, inhibiting fibrinogen binding to the platelet $\alpha_{IIb}\beta_3$ receptor, and downregulating cell adhesion molecules implicated in platelet-vascular wall adhesion, including P-selectin (Jin et al. 2005).

Early studies showed that platelet adhesion is decreased by 23 % in endothelial cells stimulated with bradykinin (100 nmol/L), a substance that can activate eNOS (Radomski et al. 1987), suggesting that endothelial $NO^{\cdot}$ exerts a key inhibitory effect on platelet aggregation in the absence of frank tissue injury. Indeed, activation of endothelial NOS (eNOS) is a key mechanism supporting $NO^{\cdot}$-dependent platelet inhibition in the intact endothelium. This occurs mainly in response to shear stress. The flow of blood through a blood vessel is heterogeneous, as velocity is highest at the center of the vessel and nearer the vessel wall. The difference in flow rate between two concentric trajectories within a blood vessel creates a shearing effect that perturbs the endothelial surface: the shear rate (cm/s) determines shear

stress, which is defined as force per unit area (dyne/cm^2) exerted on the vessel wall as a consequence of differences in blood flow velocity (Ruggeri and Mendolicchio 2007). Shear stress levels vary according to vessel caliber and orientation, but range between <12 dyne/cm^2 and 15–70 dyne/cm^2 in geometrically irregular and straight arteries, respectively (Chatzizisis et al. 2007; Gimbrone et al. 2000). Shear stress levels as low as 10 dyne/cm^2 are associated with increased levels of bioavailable NO$^{\cdot}$ (Go), which is generated through stimulation of eNOS mRNA transcription, posttranslational activation of eNOS (i.e., phosphorylation), and upregulation of eNOS-dependent NO$^{\cdot}$ synthesis (Boo et al. 2002). By contrast, elevated (>80 dyne/cm^2) or nonpulsatile shear stress levels are associated with platelet activation, initially through the induction of the glycoprotein Ib–V–IX interaction with von Willebrand Factor (vWF) (Shankaran et al. 2003).

A preponderance of data suggests that platelets express a constitutive NOS isoform and synthesize NO$^{\cdot}$ autonomously (Gambaryan et al. 2008). Freedman and colleagues demonstrated in gel-filtered platelets near-simultaneous increases in platelet aggregation and NO$^{\cdot}$ production, measured by nephelometric and microelectrode techniques, respectively, following stimulation with adenosine 5-′-diphosphate (ADP) (Freedman et al. 1997). More recently, others have demonstrated the functional effects of platelet-derived NO$^{\cdot}$ by measuring platelet levels of the adhesion molecule P-selectin and thrombosis burden in mice injected with collagen plus epinephrine. Findings from these observations support platelet-derived NO$^{\cdot}$ involvement in preventing thrombosis in vivo and suggest this may be an accessible treatment target. For example, repurposing of the β-adrenergic receptor antagonist d(L)-nebivolol has emerged recently as a potential antithrombotic therapy based on data indicating that its use in eNOS-deficient mice increases platelet levels of cGMP and platelet-derived NO$^{\cdot}$ metabolites (nitrite [NO_2^-] and nitrate [NO_3^-], NOx) and decreases embolism-associated mortality in an experimental model of femoral deep vein thrombosis (Momi et al. 2014). These data are consistent with findings from others demonstrating that d(L)-nebivolol stimulates β_2- and β_3-adrenergic receptor signaling, which results in eNOS phosphorylation at Ser1177, subsequent eNOS activation, and increased NO$^{\cdot}$ in platelets (Momi et al. 2014; Signorello et al. 2011). Directionally similar findings have also been reported for the HMG-CoA reductase inhibitor atorvastatin in experimental models of carotid arterial thrombosis, which is hypothesized to occur via upregulation of eNOS expression directly or by virtue of its antioxidant properties and, hence, diminished consumption of platelet-derived NO$^{\cdot}$ by superoxide anion ($O_2^{-\cdot}$) that generates the reactive oxidant peroxynitrite ($ONOO^-$) (Yokoyama et al. 2005).

Platelet-derived NO$^{\cdot}$ synthesis under basal conditions is reported to achieve 20 nM in vitro, although it is unresolved as to whether or not platelet inhibition occurs at these levels in the setting of vascular injury in vivo (Freedman et al. 1999). By contrast, unstable angina, acute myocardial infarction, stroke, atrial fibrillation, thrombotic microangiopathy, preeclampsia, and familial syndromes of atherothrombosis are examples of human diseases characterized by increased thrombosis in the setting of decreased endothelial NO$^{\cdot}$ (Freedman et al. 1996). eNOS knockout mice are characterized by abnormal vascular reactivity, systemic hypertension, and decreased NO$^{\cdot}$ release from platelets, with a shortening of bleeding time also noted. This latter finding is attributed to changes in bioavailable levels of tissue plasminogen activator (t-PA) and vWF, and, subsequently, disruption to the balance between coagulation, fibrinolysis, and platelet recruitment (Freedman et al. 1999; Iafrati et al. 2005). This perturbation has been shown in models of vascular injury to regulate platelet reactivity in vivo: thrombin-induced platelet aggregation is enhanced in mice pretreated with the NOS inhibitor N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) (50 mg/kg) and transfused with normal platelets, suggesting that platelet-derived NO$^{\cdot}$ alone may be insufficient to control the thrombosis response fully (Tymvios et al. 2009). Studies assessing the consequence of eNOS deficiency have yielded similar findings in cerebral (Tan et al. 2015), coronary (Knudson et al. 2005), and resistance circulations (Toda and Toda 2011), again suggesting a contribution from both endothelial and platelet-derived NO$^{\cdot}$ in control of thrombosis, although differences in model type and design have been suggested to account for some inconsistent results (Ozuyaman et al. 2005).

Endothelial or platelet-derived NO$^{\cdot}$ inhibits platelet adhesion to the endothelium through several mechanisms, including attenuation of P-selectin expression (Gries et al. 1998), inhibition of ERK/p38-dependent GP IIIb/IIa activation through protein kinase G (PKG) signaling (Roberts et al. 2009), and decreased cytosolic Ca^{2+} (Fig. 1). The latter may occur via cGMP-PKG signaling that phosphorylates phospholipase C, or through cGMP-independent mechanisms by increasing sarcoplasmic/endoplasmic reticular Ca^{2+} refilling that depletes cytosolic Ca^{2+} levels (Trepakova et al. 1999). By contrast, decreased levels of bioavailable NO$^{\cdot}$ are implicated in failure to suppress these platelet regulatory mechanisms and correlate with a heightened predilection to thrombosis (Murohara et al. 1995). Likewise, strategies to boost NO$^{\cdot}$ bioactivity are associated with thrombosis prevention. For example, Wistar rats with induced myocardial infarction and heart failure that were randomized to treatment with the novel eNOS transcription enhancer AVE9488 for 10 weeks had significantly decreased platelet surface expression of P-selectin compared to

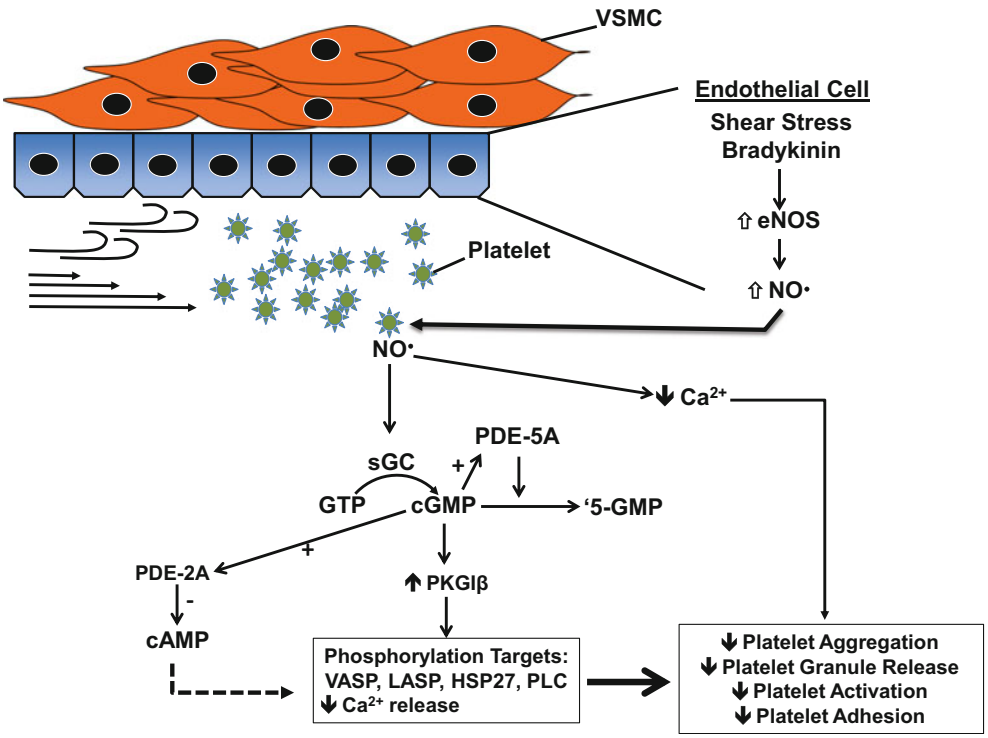


Fig. 1 The effect of nitric oxide on platelet function. Increased shear stress due to nonlaminar blood flow (*curled lines*) stimulates endothelial nitric oxide synthase (eNOS) activity in vascular endothelial cells to synthesize nitric oxide ($NO^{\cdot}$), which targets various cell types including platelets. Alternatively, eNOS-dependent synthesis of $NO^{\cdot}$ also occurs in platelets in response to increased adenosine diphosphate. In platelets, $NO^{\cdot}$ stimulates the heterodimeric enzyme soluble guanylyl cyclase (sGC), which catalyzes the conversion of guanosine triphosphate (GTP) to the second messenger cyclic guanonsine monophosphate (cGMP). Upregulation of cGMP-dependent protein kinase (GβI) in platelets promotes phosphorylation of key proteins involved in platelet inhibition, including vasodilator-stimulated phosphoprotein (VASP), Lim and SH3 protein domain (LASP), and heat shock protein 27 (HSP27). Counter-regulatory feedback mechanisms stimulated by sGC activation include phosphodiesterase type 5A (PDE-5A)-dependent hydrolysis of cGMP to 5'GMP, which is inactive, as well as PDE-2A-dependent inhibition of cyclic adenosine monophosphate (cAMP). PLC, phospholipase C; $[Ca^{2+}]_i$, intracellular calcium; VSMC, vascular smooth muscle cell; straight lined arrows represents laminar blood flow

placebo-treated rats, despite evidence of impaired NO-cGMP signaling in the pretreatment state (Schafer et al. 2009). These data complement work from others demonstrating the antiplatelet and antithrombotic effect of exogenous $NO^{\cdot}$ in biological systems in vitro and in vivo, although the benefit of pharmacological $NO^{\cdot}$ donors in reducing atherothrombotic mortality or other hard clinical end points remains less certain.

Prostacyclin

Key by-products of arachidonic metabolism relevant to platelet bioactivity include the prostanoid, thromboxane, and prostaglandins (Fig. 2). Arachidonic acid is released from the endothelial membrane following phospholipid hydrolysis by phospholipase A_2, which occurs under basal conditions, although enzymatic activity is also increased during inflammation or in the setting of atherosclerosis (Yang et al. 2006). Enzymatic activity of cyclooxygenase (COX)-1 and -2, which are constitutive and highly inducible, respectively, catalyzes the synthesis of prostaglandin by incorporating two moles of diatomic oxygen (O_2) (forming the unstable intermediary PGG_2) followed by peroxidase-mediated 2-electron reduction of PGG_2 to form PGH_2. Terminal prostanoid synthases convert PGH_2 into various prostaglandins, including thromboxane and PGI_2 that are the key species targeting platelets (Capra et al. 2014).

In 1976, Moncada and colleagues (Moncada et al. 1976) described a novel prostaglandin (PGX), which manifests antiplatelet aggregation properties and was found in high abundance in vascular endothelial cells. Later, PGX was denoted PGI_2 and was determined to be the principal prostaglandin in endothelial cells, with lesser concentrations observed in other vascular or support cells of the blood vessel. The target of PGI_2 is the prostacyclin (IP) receptor, which is a 7-transmembrane domain G-protein-coupled receptor that consists of a binding pocket that accommodates the cyclopentane ring of various PGs (Yamada et al. 2003). Murine kinetic studies using radiolabeled synthetic PGI_2

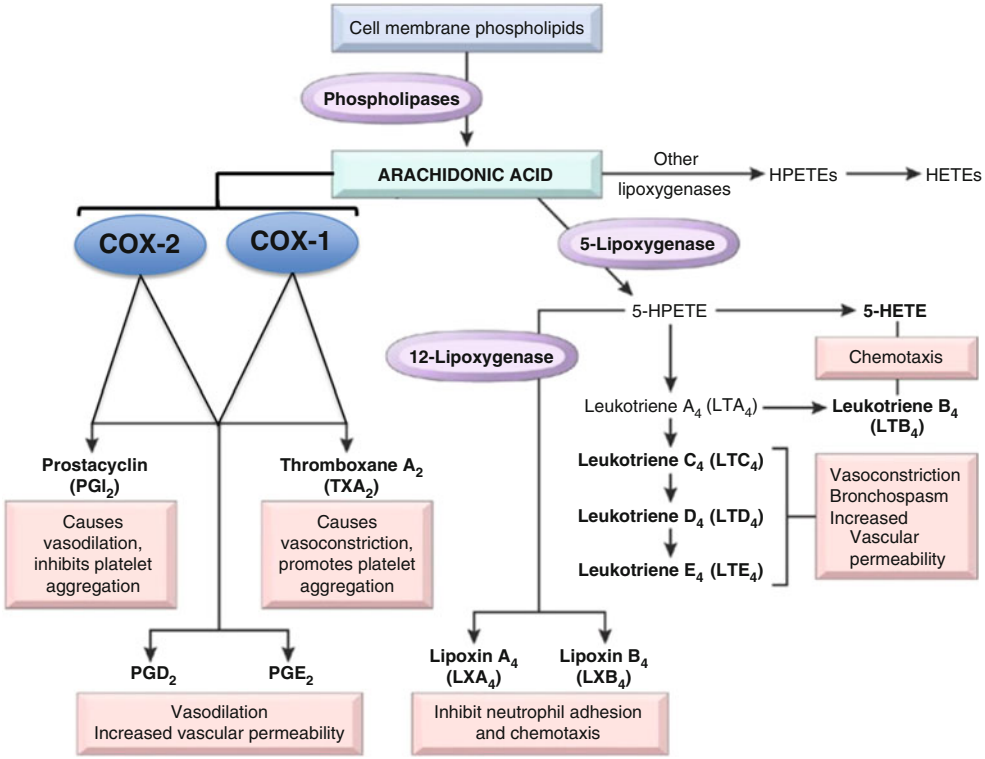


Fig. 2 Arachidonic acid metabolism. *Adapted with permission from* Robbins & Cotran's *Pathologic Basis of Disease 8th ed. Kumar V et al. (eds).* Saunders Elsevier. Philadelphia (2010)

(Iloprost) demonstrated a K_i for PGI_2-IP of 11 nM, which is akin to that for the synthetic PGI_2 cicaprost (10 nM), but substantially less than that for carbacyclin (110 nM) (Kiriyama et al. 1997). The precise structural features that influence IP binding by PGI_2 or cross-reactivity with other prostaglandins, however, remain unknown.

There is substantial overlap between factors that regulate endothelial COX-2 and mediators of cardiovascular disease, including shear stress (Healy et al. 2005), hypoxia (Schmedtje et al. 1997), thrombin (Syeda et al. 2006), and pathophysiologically elevated levels of angiotensin II (Zerrouk et al. 1998) and aldosterone (Xavier et al. 2011). Increased vascular oxidant stress, which is associated with each of these, may be one converging mechanism by which agents these affect COX-2 regulation of platelets. Under pathological conditions, vascular endothelial cells and platelets synthesize elevated levels of the reactive oxygen species (ROS) superoxide anion ($O_2^{-\cdot}$), H_2O_2, and hydroxyl anion ($^{\cdot}OH$), which in the setting of thrombosis are likely derived mainly from NAD(P)H oxidase (NOX) or xanthine oxidase enzyme activation (Marcus et al. 1977). Additional triggers of ROS release in platelets include their stimulation by collagen or eNOS uncoupling (Essex 2009; Pignatelli et al. 1998). Platelet-released $O_2^{-\cdot}$, in turn, is associated with increased availability of ADP, activation of thrombin receptor-activating peptide that inhibits thrombus dissolution, and scavenging of $NO^{\cdot}$ by $O_2^{-\cdot}$ which generates $ONOO^-$ (Essex 2009). Endothelial H_2O_2 generation stimulated by the inflammatory cytokine TNF-α increases endothelial COX-2 expression and COX-2-dependent increases in TXA_2 levels (Eligini et al. 2005) (see discussion on overlap between COX-1 and COX-2 signaling, later).

Strategies that attenuate ROS accumulation in endothelial cells or platelets may reverse this prothrombotic tendency. Pignatelli and colleagues observed an inverse, dose-dependent effect of catalase (which reduces H_2O_2 to water) on TXA_2 synthesis, platelet membrane arachidonic acid release, and inositol triphosphate (IP3) formation (Pignatelli et al. 1998). More recently, the plant derivative and antioxidant catechol (benzenediol) (1–10 μM) was demonstrated to attenuate rabbit platelet COX-2 activity and TXA_2-induced platelet activation, which correlated positively with inhibition of platelet-derived ROS accumulation (Chang et al. 2014). Others propose transcription and activation of NF-κB, a redox-sensitive transcription factor present in various vascular cells involved in thrombosis, as a unifying mechanism involved in COX-2 regulation (Schmedtje et al. 1997).

Generally, prostanoids have a short half-life (e.g., 3 s for PGI_2) and, thus, do not have sustained bioactivity in the circulation. However, PG stability is sufficient to enable physical interaction with the IP after release from the vessel wall. Stimulation of the IP by PGI_2 promotes coupling of the heterodimeric G protein subunit $G\alpha_s$, resulting in activation of adenylyl cyclase (AC) 3, AC6, and AC 17 (Dittrich et al. 2008) in platelets (Kobayashi et al. 2000). Activation of

protein kinase A (PKA) by AC-derived cAMP occurs in human platelets through dissociation of the PKA regulatory and catalytic subunits RIα, RIβ, RIIβ, Cα, and Cβ (Smolenski 2012; Rowley et al. 2011) and confers on platelets a multitude of downstream effects including inhibition of adhesion, aggregation, and granule release.

A number of PKA substrates involved in mediating platelet bioactivity have been studied. For example, phosphorylation of the small G-protein Rap1B at Ser7 or Ser179 (Subramanian et al. 2013) by PKA deactivates Rap1B and inhibits platelet aggregation. Compared to wild-type mice, *rap1b*$^{-/-}$ mice have a four-fold increase in bleeding time attributable to decreased platelet aggregation, exhibit impaired platelet reactivity in response to ADP and epinephrine (Chrzanowska-Wodnicka et al. 2005), and express an abnormal response to mechanotransduction in the endothelium (Lakshmikanthan et al. 2015).

Phosphorylation of the myosin light chain (MLC) component of the actomyosin complex is another critical target of PGI_2-PKA signaling. Although platelet activation through Ca^{2+} release is an important mechanism underlying TXA_2-mediated MLC kinase (MLCK) and MLC_{20} phosphorylation (Offermanns 2006; Wikstrom et al. 2008), PGI_2 attenuates platelet activation/aggregation through Ca^{2+}-independent mechanisms, including via RhoA. In particular, stimulation of the IP subunit $G\alpha_{13}$ is linked to Rho-specific guanine nucleotide factor activation (Hart et al. 1998), which, in turn, inactivates myosin phosphatase, MLC, and the negative regulator of MLC CPI-17 (Wikstrom et al. 2008). Collectively, these changes decrease cellular Ca^{2+} stores and limit MLCK binding of calmodulin, resulting in attenuated platelet contraction and induced platelet disaggregation (Body 1996). Alternatively, PGI_2 has been shown to induce phosphorylation of the platelet inositol 1,4,5-triphosphate ($InsP_3$) receptor in saponin-permeabilized platelets in vitro and human platelets ex vivo, which abolishes the Ca^{2+} releasing activity of $InsP_3$ (Tohmatsu et al. 1989). However, this effect does not appear to be contingent on de novo $InsP_3$-mediated Ca^{2+} release, as PGI_2-induced inhibition of $InsP_3$ (and subsequent depletion of Ca^{2+}) is observed after $InsP_3$ stimulation with thrombin (Cavallini et al. 1996).

Upregulation of PGI_2 may also target vasodilator-stimulated phosphoprotein (VASP), which is a member of the proline-rich protein family and serves as a major receptor for cAMP and cGMP in various mammalian cell types (Lincoln and Cornwell 1993; Aszodi et al. 1999). Compared to normal platelets, transgenically modified VASP$^{-/-}$ platelets demonstrate a decrease in time necessary for collagen-induced aggregation (50 % maximum aggregatory response: 106 ± 4 vs. 89 ± 4 s, $P < 0.003$), which parallels differences in aggregation inhibition induced by low cGMP concentrations (77.8 vs. 21.6 %, $P < 0.002$). Posttranslational modifications of VASP by PKA include its phosphorylation at Ser157, Ser239, and Thr278, which is used to assess VASP activity experimentally. For example, multidrug resistance protein-4 (MRP4), which regulates cyclic nucleotides by promoting their extracellular transit, was recently demonstrated to prolong bleeding time and decrease platelet aggregation in response to vascular injury through its redistribution of cAMP to dense granules and upregulation of phosphorylated VASP (Decouture et al. 2015).

It is important to note that cross-talk is observed between signaling pathways that mediate the effects of TXA_2 and PGI_2, despite the opposing functions of these prostaglandins. For example, Cheng and colleagues observed that the platelet-stimulating effects of TXA_2 are inhibited by PGI_2-IP signaling. The fact that TXA_2 receptor knockout mice are prone to bleeding (Thomas et al. 1998) is likely attributed to the effect of PGI_2-IP on platelet–endothelial interactions through MLC, $InsP_3$, and VASP (Cheng et al. 2002). Furthermore, it is hypothesized that a thrombotic tendency occurs under conditions in which COX-1 activity is not offset by the antiplatelet/vasodilatory effects of COX-2 in the vascular endothelium (Pratico and Dogne 2005). This hypothesis, which is supported by some mechanistic data (Grosser et al. 2006), emerged largely based on a number of clinical studies involving the use of selective COX-2 inhibitors for the treatment of rheumatological disease. Data from these trials indicated a signal toward increased risk of cardiovascular thrombotic events in patients treated with selective COX-2 inhibitors. In one meta-analysis (Kearney et al. 2006) of 31,129 patients, for example, selective COX-2 inhibitor celecoxib use was associated with a 50 % increase in the relative risk of myocardial infarction (0.6 %/year) compared to nonselective COX-1/-2 inhibitors, prompting a black box warning for the use of celecoxib with respect to cardiovascular disease event risk. More recent data, however, suggest that celecoxib at moderate doses is non-inferior to naproxen and ibuprofen with respect to safety (Nissen et al. 2016).

EctoADPase/CD39

Ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases) comprise a family of endothelial cell membrane glycoproteins that use Ca^{2+} or Mg^{2+} as cofactors to hydrolyze proinflammatory/thrombotic extracellular 5′-di- and triphosphatases (i.e., nucleotides). Among this protein class, CD39 (NTPDase1; also denoted apyrase, ecto-ATPase, ecto-ADPase, nucleotide phosphohydrolase, or ATP pyrophosphohydrolase) is the principal vascular ENTPDase, although it has also been identified in human placenta, lung, skeletal muscle, kidney, and heart tissues (Kaczmarek et al. 1996). Endothelial CD39 has no direct physical interaction with platelets but mediates platelet

aggregation by metabolizing ADP and inhibiting ADP-dependent platelet activation (Marcus et al. 1997). This effect was observed initially by Marcus and colleagues, who demonstrated that platelet reactivity could be reversed within 60 s in CD39-trasfected COS cells stimulated with 10 μM ADP (Marcus et al. 1997). In Chinese hamster ovary cells, the Km of soluble CD39 for ADP and ATP are 5.9 μM and 2.1 μM, respectively, while the catalytic efficiency (k_{cat}/K_m) is 1.2×10^8 min^{-1} M^{-1} for both ADP and ATP (Gayle et al. 1998). These binding kinetics, which favor association of CD39 with ADP, may account for autonomous endothelial regulation of platelet bioactivity even under conditions in which $NO^{\cdot}$ or PGI_2 are diminished. Indeed, recombinant endothelial CD39 blocks ADP-induced platelet aggregation in vitro and inhibits collagen-induced platelet reactivity.

There are also accumulating data to support the importance of CD39 in the pathogenesis of thrombotic vascular disease clinically. In patients with angiographically proven epicardial coronary artery disease (CAD), the ratio of plasma ectonucleotidase ADPase-to-ATPase activity is decreased compared to controls (El-Omar et al. 2005), indicating that endogenous CD39 is impaired in CAD patients and that CD39 may hold promise as a potential treatment target to decrease clinical events in patients with atherosclerotic disease. The HMG-CoA-reductase inhibitors simvastatin or cerivastatin have also been shown to improve the metabolism of exogenous ADP and attenuate platelet activation fully despite treatment with thrombin in vitro (Kaneider et al. 2002), while exercise is linked to increased endogenous CD39 expression and decreased platelet aggregation in patients (Coppola et al. 2005).

S-Nitrosylation

Early observations indicating that the half-life of $NO^{\cdot}$ in plasma is substantially longer than in aqueous solution (6 vs. 0.1 s) suggested the existence of $NO^{\cdot}$ molecular adducts that protect $NO^{\cdot}$ metabolism in vivo (Keaney et al. 1993). Subsequently, it was determined that $NO^{\cdot}$ may react with transition metals (Cu^{+}, Cu^{2+}, Fe^{2+}), other free radicals, or molecular oxygen to generate various nitrogen oxide species (N_2O_3 or $ONOO^{-}$), and that the reactive nitrogen derivative of $NO^{\cdot}$ forms a covalent bond with the sulfhydryl group of cysteine to generate *S*-nitrosothiols (RSNOs) (Fig. 3) *S*-nitroso-glutathione reductase (GSNOR) and

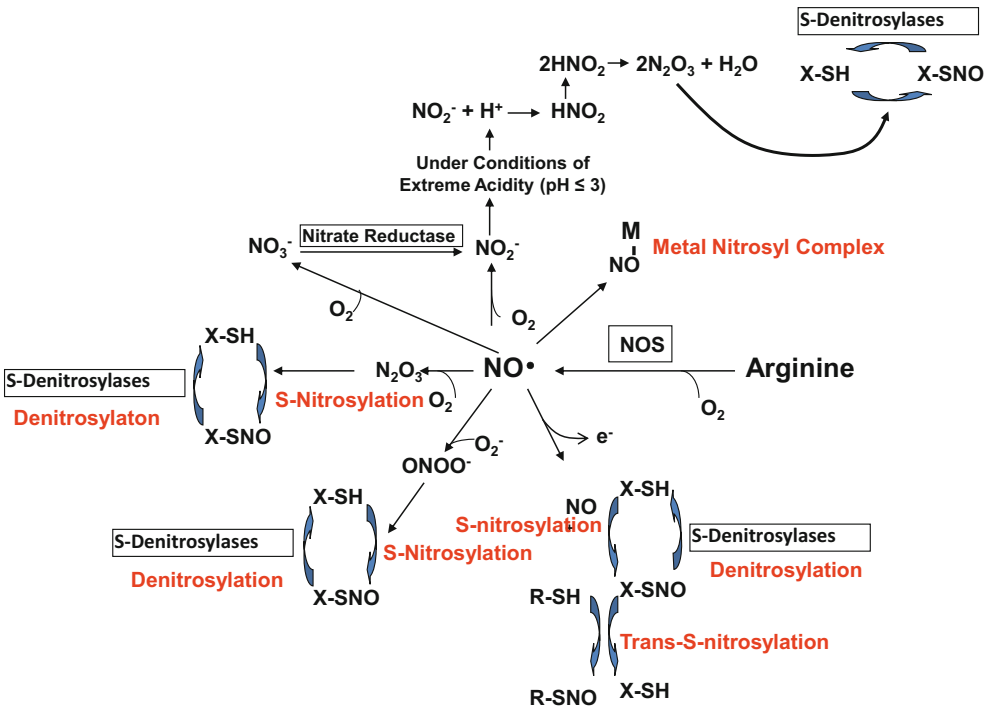
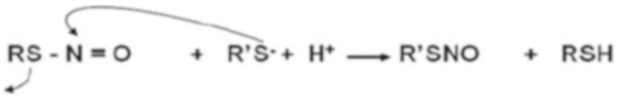


Fig. 3 *S*-Nitrosylation reactions. Nitric oxide ($NO^{\cdot}$) synthesized from L-arginine by nitric oxide synthase (NOS) may bind to transitional metals (NO-M), undergo oxidation in the presence of molecular oxygen (O_2), or interact with superoxide anion ($O_2^{\cdot-}$) to form peroxynitrite ($ONOO^{-}$). In the presence of O_2, $NO^{\cdot}$ may be converted to nitrosonium (NO^{+}), an electrophile that interacts with nucleophilic thiols to form *S*-nitrosothiols (SNO). Additionally, under conditions of extreme acidity (pH $\leq$ 3), protonation of $NO^{\cdot}$ yields the formation of nitrous acid (HNO_2), which is a reactant for the synthesis of the nitrosylating agent nitrogen trioxide (N_2O_3). *S*-Nitrosothiol denitrosylation may occur enzymatically or through nonenzymatic exchange with free thiols. *Adapted from* Maron BA, et al. Antioxid Redox Signal 2013;18(3):270–287

thioredoxin reductase (TrxR) are two key denitrosylating enzymes that induce NO· release from SNOs. The mechanism by which this occurs involves a series of intermediate reactions including, in the case of TrxR, nucleophilic attack of the target cysteine by the N-terminal Cys thiolate ($R'S^-$) of TrxR, the formation of an intermolecular disulfide bond, and subsequent liberation of HNO and NO· [reviewed in detail in (Maron et al. 2013)].

S-nitroso-human serum albumin (SNO-SA), *S*-nitrosocysteine, and *S*-nitrosohemoglobin are three principal RSNOs discovered in plasma that protect NO· from oxidation and allow for paracrine effects of NO· on targets distal to the site of NO· synthesis. *S*-nitrosoglutathione (GSNO) is the most abundant intracellular RSNO and has been implicated in platelet synthesis and bioactivity. Megakaryocyte-derived platelet generation is sevenfold greater in the presence of GSNO as compared to thrombopoietin (Battinelli et al. 2001), while GSNO fully inhibits platelet aggregation (Liu et al. 1998) and is associated with impaired platelet adhesion to collagen in vitro (Irwin et al. 2009).

Protein disulfide isomerase (PDI) is believed to facilitate intracellular NO· entry into platelets through trans-*S*-nitrosylation (Maron et al. 2013; Zai et al. 1999). In trans-*S*-nitrosylation, the nitrogen of an *S*-nitrosothiol is the substrate for nucleophilic attack by the thiolate ($R'S^-$) of R'SH according to the following equation (Maron et al. 2013):



Trans-*S*-nitrosylation of GSNO and *S*-nitrosocysteine suppress thrombosis by virtue of cGMP-mediated inhibition of platelet aggregation, granule secretion, and fibrinogen binding (Gordge and Xiao 2010; Mendelsohn et al. 1990). Importantly, GSNO appears to promote these effects without affecting vascular tone, indicating selective delivery of NO· to platelets. In fact, although detectable levels of PDI have been reported for smooth muscle cells and endothelial cells (Xiao and Gordge 2011), expression and provoked activity of PDI is substantially greater on the cell membrane surface of platelets where its activation hinges on conformational changes to platelet glycoprotein GP1bα (Hogg et al. 1997). Furthermore, GSNO-mediated delivery of NO· is greater in platelets than in vascular cells and can be inhibited to a greater extent in the presence of the thiol isomerase inhibitor phenylarsine oxide (Xiao and Gordge 2011). Another protein involved in hemostasis that is the target of *S*-nitrosylation is tissue plasminogen activator (t-PA): SNO-t-PA at Cys83 increases fibrinogen-stimulated t-PA activity by 4.4-fold as well as rate and extent of platelet inhibition by 50 % over unmodified t-PA (Stamler et al. 1992).

Using RSNO-based technology has emerged as an attractive approach to manipulating the platelet–vascular interaction for therapeutic purposes. Yang and colleagues reported on a NO-catalytic bioactive coating developed through covalent binding of 3,3'-dipropionic acid diselenide, (SeDPA). They demonstrated that the addition of GSNO sustained inhibition of collagen-stimulated platelet activation and adhesion to vascular smooth muscle cells for 30 days in vitro (Yang et al. 2015). Other systems have been tested in preclinical scenarios, including polythiolated S-NO-bovine serum albumin (Marks et al. 1995) and the polymeric delivery of pH-activated spermine/NO· for sustained NO· release. In one study using the rat ileofemoral balloon model of vascular injury, vessels injected with spermine/NO· engineered for drug delivery over 14 days demonstrated a decrease in neointimal proliferation and an increase in cGMP levels by 69 % and 64 % at study completion, respectively, compared to control. Similar beneficial effects of spermine/NO· have also been reported in experiments on injured cerebral (Scheckenbach et al. 2006) and pulmonary vascular beds (De Witt et al. 2001).

Special Considerations in the Pulmonary Vasculature

A number of important differences distinguish the pulmonary from systemic circulations with respect to vascular wall regulation of platelet function. The parallel circuitry of the pulmonary vascular system produces a high-flow and low resistance circulation, and, therefore, pulmonary artery atherosclerotic vascular injury is uncommon in the absence of *severe* pulmonary hypertension (Maron and Loscalzo 2013). Nonetheless, vascular inflammation, endothelial dysfunction, vascular endothelial and smooth muscle cell apoptosis resistance, and abnormal fibrillar collagen deposition underlie the plexogenic vasculopathy of pulmonary arterial hypertension (PAH), which is also characterized by platelet-rich thrombi in distal pulmonary arterioles. In addition to PAH, chronic thromboembolic pulmonary hypertension (CTEPH) is an important clinical phenotype that generally occurs after luminal pulmonary embolism but is characterized by in situ thrombosis of large and segmental pulmonary arteries and small-vessel arteriopathy in the setting of sustained platelet overactivation (Lang and Madani 2014).

In PAH or CTEPH patients, increased thrombus susceptibility is associated with a decrease in pulmonary endothelial NO·, which, in turn, appears to be a consequence of decreased eNOS expression (Giaid and Saleh 1995); altered levels of key substrates required for NOS-dependent NO· synthesis, such as L-arginine; eNOS uncoupling due to depletion of the eNOS cofactor tetrahydrobiopterin (BH_4) (Xu et al. 2004); and/or oxidation of NO· in the setting of increased vascular ROS accumulation (Maron et al. 2013). Monogenetic causes of PAH, including inherited (loss of

function) BMPR-2 mutations, are also associated with diminished eNOS activity through inhibition of normal BMP2- and BMP4-mediated signal transduction (Klinger et al. 2013). Gangopahyay and colleagues demonstrated that in cultured pulmonary artery endothelial cells (PAECs), the BMPR-2 ligands BMP2 and BMP4 promote PKA-dependent phosphorylation and activation of eNOS. In that study, molecular inhibition of BMPR-2 in cultured PAECs uncoupled BMP2/4-eNOS signaling, while BMP2/4 failed to stimulate eNOS in PAECs isolated from patients with BMPR-2-dependent PAH (Gangopahyay et al. 2011).

Although a genetic predisposition to hypercoagulability has been proposed to account for CTEPH incidence, polymorphisms in thrombogenic genes, such as Factor V Leiden (G20210A), plasminogen activator inhibitor-1 4G/5G, tissue plasminogen activator C7351T, Factor XIII G100T, or fibrinogen Bβ G455A, are identified in only a minority of patients (Suntharalingam et al. 2008). Furthermore, no significant differences are observed between platelet-poor and platelet-rich plasma from CTEPH patients with respect to levels of tissue-type plasminogen activator (t-PA) and type 1 plasminogen activator inhibitor (PAI-1), and CTEPH patients do not differ significantly from healthy controls with respect to activity levels of these prothrombotic mediators (Olman et al. 1992). From these collective observations, increased attention has turned toward impaired vascular suppression of platelet activation in the phase following initial pulmonary embolism as a critical determinate of disease progression.

Compared to sex-matched healthy controls, postendarterectomy thrombus specimens or plasma from CTEPH patients express increased levels of key inflammatory cytokines, including interleukin (IL)-6, IL-8, IL-10, monocyte chemoattractant protein-1, and interferon-γ-induced protein-10 (Zabini et al. 2014), suggesting a persistent inflammatory milieu underlying the platelet–endothelial interface in CTEPH patients. In turn, increased pulmonary vascular inflammation is linked to dysregulated angiogenesis and impaired endogenous thrombolysis. In an experimental model of pulmonary venous thromboembolism, for example, delayed thrombus resolution is observed in mice expressing an endothelial cell-specific deletion of the endothelial growth factor receptor 2 (VEGF-2R)/kinase insert domain protein receptor deletion (Alias et al. 2014). Others have observed an increase in the abundance of collagen-secreting cells from endarterectomy samples and contend that this potentiates endothelial injury and propagates thrombosis formation in situ (Yao et al. 2009). Recently, it has also been proposed that endothelial injury in CTEPH occurs due to an increase in vasa vasorum density, which serves as a reservoir for migrating endothelial cells, proliferation, and recanalization of thrombus (Kimura et al. 2001). These changes are maladaptive, however, and are linked mechanistically to a prothrombotic vascular surface, possibly related to the upregulation of angiostatic and platelet-activating factors, including increased PF4 and collagen type I (Zabini et al. 2012).

In animal models of pulmonary venothromboemoblism and in CTEPH patients, hypoxia and mediators of inflammation (particularly IL-6) diminish pulmonary endothelial cGMP bioavailability due, in part, to increased phosphodiesterase-mediated hydrolysis of cGMP to its inactive metabolite 5'-GMP (Evgenov et al. 2004). Diminished cGMP, in turn, disrupts the cellular $[Ca^{2+}]_0$ -to- $[Ca^{2+}]_i$ gradient and increases $[Ca^{2+}]_i$ that impairs endothelial barrier function and promotes vascular injury, edema, and thrombogenicity (Moore et al. 1998). Hypoxia also increases pulmonary artery endothelial vWF expression through inhibition of the nuclear repressor nuclear factor-IB association with the vWF promoter (Mojiri et al. 2013), and appears to suppress leukotriene metabolism in PAH in vivo. Hypoxia-PAH rats express increased platelet $\alpha_{IIb}\beta_3$, as well as endothelial expression of P-selectin and platelet factor-4 (PF4), which, collectively, are prevented by treatment with the 15-lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) (Shen et al. 2015) or the COX-2 inhibitor diethylcarbamazine (Al-Husseini et al. 2015). These findings are consistent with work demonstrating that overexpression of 5-lipoxygenase results in abnormal endothelial cell proliferation (Zhang et al. 2002; Walker et al. 2002).

Biosynthetics in Platelet–Vascular Wall Interactions

There has been substantial progress in the development of biosynthetics and novel platforms in silico for clarifying the physical and molecular factors underlying platelet–endothelial interactions. This includes the emergence of micro/nanostructure- and nitinol-based surfaces to study platelet-vascular function ex vivo and in vivo (Huh et al. 2010; Nozaki et al. 2015), the development of $NO^{\cdot}$-releasing stent prototypes (Yang et al. 2015), and synthetic vascular grafts engineered to improve endothelial cell binding that reduce thrombotic potential (Solouk et al. 2015).

Although a number of vascular stent coatings have been tested or are approved for use in clinical practice, including diamond-like carbon, titanium-nitride-oxide, and plasma-activated coatings among others (Ma et al. 2007), endothelialization extent and rate associated with these synthetics remain suboptimal and are believed to cause some forms of stent failure observed clinically. Use of novel surface modifications that leverage enhanced hydrophilicity, such as SiCOH, n-butyl methacrylate, and phosphorylcholine (Zhang et al. 2014), as well as apyrase (ADPase) and other biomolecules that inhibit platelets

directly, frames the next generation of biosynthetic stents with increased antithrombogenic potential (Zhang et al. 2014). Modification of surfaces with pendant tags that attract endothelial cells as a method to promote endothelial cell adherence has also gained attention recently. A polycarbonate urethane surface coated with polyethylene glycol, pentafluorophenyl methacrylate polymer, and Cys-Ala-Gly peptide tagged with allyl amine molecules, for example, has been shown to generate a hydrophilic surface favorable for promoting adhesion of functional endothelial cells and inhibiting platelet adhesion when exposed to platelet-rich plasma ex vivo (Khan et al. 2015).

To offset the limitations of studying vascular function under traditional, static conditions, some investigators have turned to three-dimensional models that aim to recapitulate physiologic conditions in silico. Huh and colleagues developed a human lung-on-a-chip microdevice, which affords dynamic study of the alveolar–capillary interface under dynamic conditions, including contemporaneous analysis of blood flow and mechanical changes associated with ventilation (Huh et al. 2010). This model has been used to test the effect of prothrombin, IL-2, and fibrinogen on endothelial fibrin formation in the setting of pulmonary edema-associated alveolar injury. Their findings, which demonstrate that even physiological mechanical force potentiates the hazardous effect of IL-2 on fibrin clot formation, provide proof-of-concept for the study of an integrative physiological system, and have important implications for analyzing endothelial–platelet interactions in understudied forms of thrombotic disease.

Conclusions

Suppression of platelet activation is a cornerstone feature of normal endothelial function and is critical for maintaining blood flow. Endothelial synthesis of $NO^{\cdot}$ induced by blood flow, endogenous release of PGI_2 in response to oxidant stress or inflammation, and endothelial CD39 that inhibits ADP-induced platelet activation are key mechanisms that define vascular regulation of thrombosis. Differences between the systemic and pulmonary vascular circuits in flow rate and design inform platelet pathobiology and the attendant clinical pathophenotypes that are commonly observed in each circulation. Gains in biosynthetic technology and the development of dynamic models that allow for studying the interaction between vascular cell types, blood flow, and vascular injury on an organ level are anticipated to further our understanding of the blood vessel–platelet relationship. One important outstanding goal in endothelial–platelet biology is the generation of novel device therapies that treat vascular dysfunction effectively in patients but with hemorrhagic risk.

Take Home Messages

1. Nitric oxide, thromboxane, and prostaglandins are important endogenous regulators of platelet bioactivity.
2. Increased vascular oxidant stress is associated with a prothrombotic phenotype.
3. Platelet aggregation can be inhibited indirectly through metabolism of ADP by ENTPDases.
4. In the pulmonary circulation, hypoxia and inflammation stimulate platelet–vascular interactions.
5. Biosynthetic systems are increasingly used to study the properties of platelet adherence to the blood vessel wall.

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Platelet–Leukocyte Interactions

Virgilio Evangelista, Licia Totani, Angelo A. Manfredi, and Norma Maugeri

Abstract

The current vision of the mechanisms involved in the hemostasis, inflammation, and innate immunity is based on the intricate interplay between platelets and leukocytes. However, were necessary about three decades to abate the paradigm in which leukocytes are involved only in the immune response and platelets only in the hemostasis. In this chapter, we try to illustrate the clinical and experimental evidences of the wealthy cross-talk between platelets and leukocytes that support real insight into the function of these cells.

Introduction

The cross-talk between platelets and leukocytes is a hallmark of thrombotic and inflammatory processes. Platelets perform diverse immune-related functions such as interacting with neutrophils (Zarbock et al. 2007) and monocytes (van Gils et al. 2009). The interaction depends on direct contact via specific adhesive ligand–receptor couples and through the generation of soluble active moieties (Semple et al. 2011). Platelets, whose primary function is the surveillance of vascular integrity and the initiation of a hemostatic plug in the case of vessel damage, also rely on the interaction with leukocytes to guard vessels against infective agents. These homeostatic interactions may, however, also contribute to tissue damage in a wide variety of acute and chronic inflammatory diseases. The cross-talk between platelets and leukocytes may result in reciprocal activation and inhibition which is likely to fine tune the eventual outcome of the inflammatory process, determining health or disease.

This chapter illustrates the basic molecular mechanisms that underlie platelet–leukocyte communication, the pro-/antithrombotic and inflammatory interactions, the clinical features associated with the cross-talk between platelets and leukocytes, and the experimental evidence that support novel pharmacological approaches targeting platelet–leukocyte interactions.

Cross-Talk Between Platelets and Leukocytes

In physiological conditions, flowing leukocytes reach sites of injury and infection via a well-regulated multistep process. They initially tether to and roll along the vessel wall. This is followed by the firm adhesion to activated endothelial cells and eventual diapedesis. These events involve various interacting ligand–receptor pairs in an adhesion cascade. The activated endothelial cells express P- and E-selectins, both recognized by leukocytes via the constitutively expressed counter-receptors, P-selectin glycoprotein ligand 1 (PSGL-1) and E-selectin ligand 1 (ESL-1) (Hidalgo

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et al. 2009; Maugeri and Manfredi 2015; Maugeri et al. 2012a, 2014a; Sreeramkumar et al. 2014; Nacher and Hidalgo 2011; Campana et al. 2014).

Moreover, leukocytes can be recruited at the site of endothelial injury and transmigrate through compromised vessels by the interaction with adherent platelets. The first evidence of platelet–leukocyte interaction during hemostasis was provided by Giulio Bizzozero in 1882 (Mazzarello et al. 2001). More than 100 years were required to confirm Bizzozero's observations: adherent platelets at the site of a damaged vessel wall promoting leukocyte rolling, arrest, and transmigration across platelet thrombi through the sequential action of P-selectin and the ß2-integrins (Manfredi et al. 2015; Ramirez et al. 2014). Conversely, leukocytes aggregated with activated platelets promote thrombus formation (Evangelista et al. 2007; Furie and Furie 2008).

Adhesive Receptors

Adhesion of platelets and leukocytes depends essentially on selectins and integrins. P-selectin expressed on activated platelets is recognized by PSGL-1 on the surface of resting leukocytes. After ligation, PSGL-1 triggers activation-dependent conformational changes of the β2 integrins, mainly CD11b/CD18 (formally αMβ2 and also called Mac-1), which mediates firm adhesion of the neutrophils and monocytes (Gardiner et al. 2001; Gawaz et al. 1998; Nacher and Hidalgo 2011; McEver and Cummings 1997; Totani and Evangelista 2010).

P-selectin—formally CD62P—originally described as GMP-140 (granule membrane protein 140 kDa) (Johnston et al. 1989) and as PADGEM (platelet activation-dependent granule external membrane protein) (Larsen et al. 1989; Hamburger and McEver 1990) is a type-I membrane protein containing an N-terminal C-type lectin domain, an epidermal growth factor (EGF)-like motif, a series of short consensus repeats, a transmembrane domain, and a cytoplasmic domain. P-selectin is stored as an integral protein of the membrane of α-granules in platelets and Weibel–Palade bodies in endothelial cells (Furie and Furie 1995). Upon cellular activation, fusion of the granules with the external membrane exposes P-selectin at the cell surface (McEver and Cummings 1997).

PSGL-1 (Sako et al. 1993; McEver and Cummings 1997), constitutively expressed on the surface of neutrophils, monocytes, dendritic cells, and subclasses of lymphocytes is an homodimeric mucin molecule bearing multiple *O*-glycans on serine and threonine residues, which displays high affinity for P-selectin. The physiologic role of P-selectin/PSGL1 axis is the recruitment of leukocytes to reach inflamed tissues (Fig. 1). Indeed, the rapid association/dissociation rates for P-selectin/PSGL-1 interactions are fundamental for initial tethering and rolling of leukocytes on P-selectin surfaces (offered by the activated endothelium or activated adherent platelets) under conditions of flow (Varki 1997; McEver and Cummings 1997; Yang et al. 1999).

As a consequence, leukocytes reach sites of injury and infection (Rouhiainen et al. 2000; Maugeri et al. 2014a; Campana et al. 2014). After having tethered to and rolled, they firmly adhere to activated endothelial cells and eventually diapedese. Mac-1 and LFA-1 (formally αLβ2 and also called CD11a/CD18) mainly mediate firm adhesion of neutrophils and monocytes to activated platelets. Activated Mac-1 recognizes at least three counter-receptors exposed on the platelet surface: fibrinogen bound to the platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) (Weber and Springer 1997), GPIbα (Simon et al. 2000a), and the junctional adhesion molecule-3 (JAM-3) (Santoso et al. 2002). Intercellular adhesion molecule-2 (ICAM-2), a member of the immunoglobulin (Ig) superfamily of adhesion molecules constitutively expressed by platelets, is probably the principal LFA-1 ligand expressed on the platelet surface (Diacovo et al. 1994).

While P-selectin/PSGL-1 interaction is critical for activated platelet/leukocyte adhesion (Evangelista et al. 2003; Manfredi et al. 2010; Totani and Evangelista 2010), resting platelets can also interact with activated leukocytes through the contact of platelet glycoprotein GPIbα with the activated Mac-1 (Simon et al. 2000a). Platelet glycoprotein GPIbα is the counter-receptor of the A1 domain of VWF, which is exposed by subendothelium after vascular injury (Ruggeri 2003; Colombatti and Bonaldo 1991). The GPIbα leucine-rich repeat and COOH-terminal flanking regions bind to the I domain present on Mac-1 (Colombatti and Bonaldo 1991; Simon et al. 2000a) that is homologous to the VWF A1 domain (Li et al. 1998; Simon et al. 2000a). This interaction offers an additional mechanism through which platelets adherent to, and become immobilized on, compromised vessel walls to support firm adhesion and, eventually, transmigration of neutrophils through the injured vessel wall (Simon et al. 2000a) (Fig. 1).

GPIbα also serves as a counter-receptor for P-selectin expressed on activated endothelium. This interaction represents another important role of platelet adhesion, where the same receptor (GPIb–IX–V) can support platelet adhesion to endothelial cells under the appropriate conditions of endothelial activation and to the subendothelial matrix when the endothelial cells have been removed by injury. Indeed, platelets rolled on activated

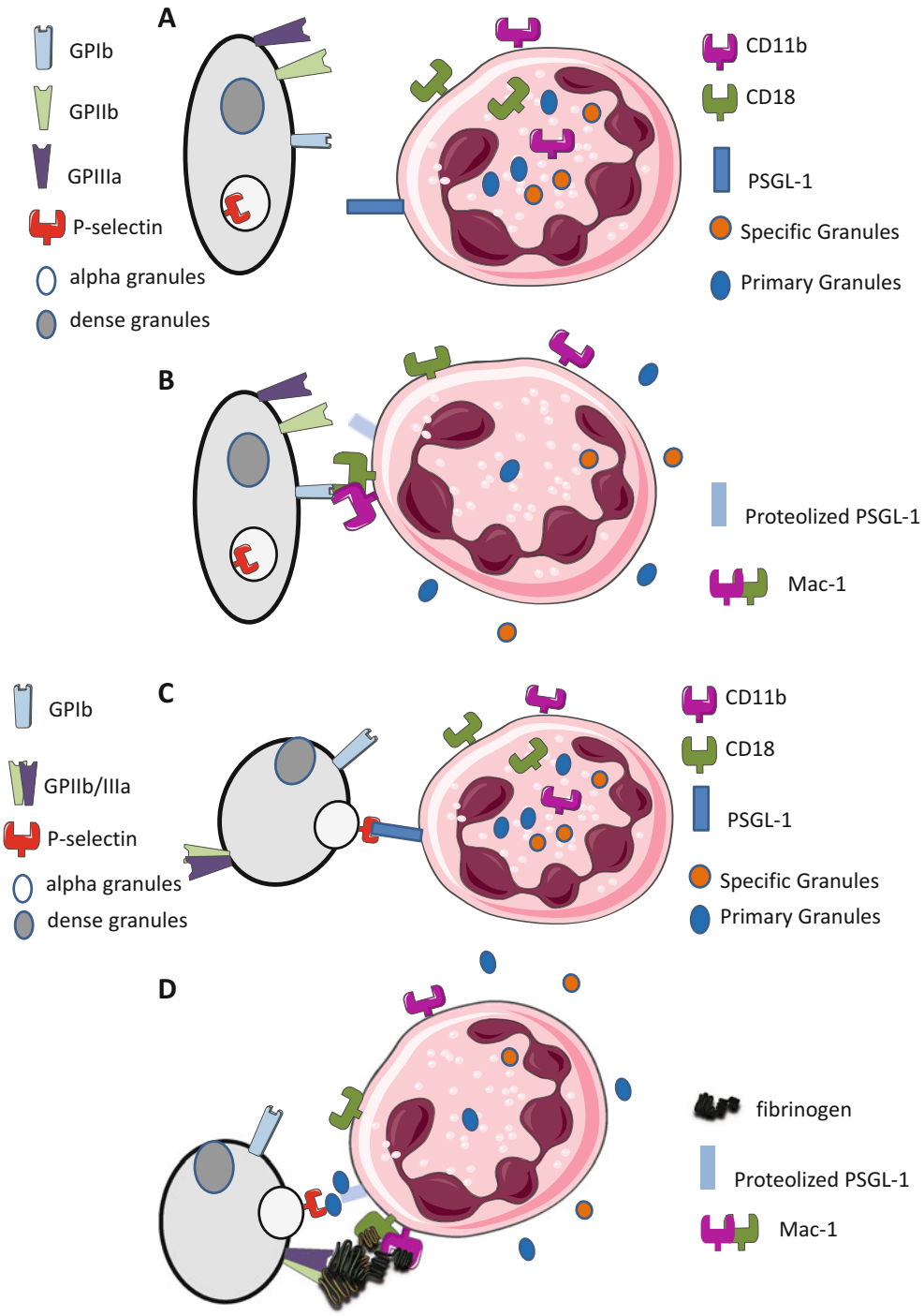

Fig. 1 Adhesive interactions between platelets and neutrophils. (**a**) Resting platelets do not interact with resting neutrophils. (**b**) Platelet GPIb recognizes the I domain expressed by activated Mac-1 (activated neutrophils). (**c**) Activated platelets express P-selectin that is recognized by neutrophils PSGL-1. (**d**) The P-selectin/PSGL-1 cross-talk induces neutrophil degranulation, degrades PSGL-1 (by the proteases derived from primary granules), and upregulates Mac-1. In this condition, Mac-1 recognized the fibrinogen bound to the platelet GPIIb–IIIa to promote the firm adhesion

endothelium, a phenomenon exquisitely dependent on both P-selectin and GPIbα (Romo et al. 1999). Unlike the P-selectin interaction with PSGL-1, the interaction with GPIbα required neither calcium nor carbohydrate core-2-branching or α(1,3)-fucosylation. Thus, injured vessel wall offers two different mechanisms to platelet interaction through GP Ib–IX–V complex: platelet attachment to both subendothelium (via vWF) and activated endothelium (via P-selectin) (Romo et al. 1999).

Soluble Mediators

Released from Platelets

Together with the hemostatic factors, platelets contain and release, upon activation, an array of mitogenic and angiogenic factors and various bioactive molecules such as platelet factor-4 (PF-4, CXCL4), β-thromboglobulin, (β–TF, CXCL7) which is converted to the CXC chemokine NAP-2 (CXCL7) by neutrophil cathepsin G, ENA-78 (CXCL5), and

Table 1 Soluble molecules released by platelets that can modify leukocyte responses

Mediator	Function	Target
CXCL1 (GROα)	Chemokine	Neutrophils
CXCL4 (PF4)	Chemokine	Neutrophils
CXCL7 (NAP2)	Chemokine	Neutrophils
CCL2 (MCP1)	Chemokine	Monocytes
CCL3 (MIP1α)	Chemokine	Eosinophils, basophils, monocytes, NK lymphocytes, dendritic cells
CCL5 (RANTES)	Chemokine	Eosinophils, basophils, monocytes, NK, dendritic cells, T lymphocytes
CCL7 (MCP3)	Chemokine	Basophils, monocytes, NK lymphocytes, dendritic cells
CCL17 (TARC)	Chemokine	T lymphocytes
CD40L	Costimulatory molecule	Monocytes, B lymphocytes, endothelial cells, dendritic cells
TREM1 ligand	Costimulatory molecule	Monocytes, neutrophils, dendritic cells
TGFβ1	Growth factor	Monocytes, neutrophils, T and B lymphocytes
PDGF	Growth factor	Monocytes macrophages, T lymphocytes
HMGB1	Chemokine	Neutrophils, monocytes, macrophages, dendritic cells

GROα (CXCL1). They also release CC chemokines including RANTES (CCL5), MCP-1 (CCL2), MIP-1α (CCL3), TARC (CCL17) and a number of miscellaneous signals with immunoregulatory properties such as the CD40L, triggering receptor expressed on myeloid cell-1 (TREM-1) ligand, transforming growth factor (TGF) β1, and the prototypical Damage-Associated Molecular Patterns (DAMPs), and high mobility group box 1 (HMGB1) (Rouhiainen et al. 2000) (Maugeri et al. 2014a, b, c; Baldini et al. 2012, 2014; Campana et al. 2014; Manfredi et al. 2015; Ramirez et al. 2014). These signals are in resting platelets stored in alpha granules or in other still uncharacterized cytoplasmic compartments (Table 1).

CXCL4 and CXCL7 are among the most abundant proteins contained in platelet α-granules. There is evidence that CXCL4 induces neutrophil activation, causing β2-integrin-mediated adhesion to endothelial cells and exocytosis (Kasper et al. 2004, 2006). CXCL4 also induces monocyte phagocytosis and respiratory burst (Pervushina et al. 2004). CXCL4 seems to contribute to leukocyte activation by the interaction with other chemokines, thus finely tuning the bioactivity of different chemokines (Proudfoot et al. 2003).

The platelet chemokine CXCL7 derives from the pro-platelet basic protein present in megakaryocytes. As the result of proteolytic modifications (Walz and Baggiolini 1990; Walz et al. 1990) it is transformed in connective tissue-activating peptide-III (CTAP-IIIβ–TG and neutrophil activating peptide-2 (NAP-2). The neutrophil CXC receptor 1 and 2 are potential receptors for NAP-2 and stimulate chemotaxis (Walz et al. 1989).

CCL5 (RANTES) (Klinger et al. 1995) promotes leukocyte migration (Kameyoshi et al. 1992; von Hundelshausen et al. 2001; Gilat et al. 1994) via the adherent activated platelets to reach the surrounding tissues. This mechanism might account for the role of platelet-derived CCL5 in the progression of atherosclerosis in experimental murine models (Huo et al. 2003; Schober et al. 2002; von Hundelshausen et al. 2005). Consistent with this concept, treatment with specific blocking peptides against CCL5–CXCL4 heterodimers attenuate monocyte recruitment and reduce development of atherosclerotic lesions in mice (Koenen et al. 2009). Other bioactive molecules such as CCL2 (monocyte chemotactic protein-, MCP-1) (Gosling et al. 1999; Boring et al. 1998; Dawson et al. 1999), CCL3 (macrophage inflammatory protein [MIP]-1α) (Klinger et al. 1995), and CCL17 (thymus and activation-regulated chemokine [TARC]) (Fujisawa et al. 2002) also contribute to the development and stabilization of atherosclerotic lesions (Gu et al. 1998; Gawaz et al. 1998).

Platelets contain and release CD154 (known as CD40L), an inflammatory molecule that reacts with the CD40 present on monocytes, dendritic cells, and vascular endothelium (Grewal and Flavell 1998; Schonbeck and Libby 2001). CD154 induces the expression of tissue factor by endothelial cells (Henn et al. 1998) and monocytes (Sanguigni et al. 2005) and has been proposed as a key player in the development and progression of cardiovascular disease (Lutgens and Daemen 2002).

TGFβ is present in large amounts in platelet α granules, and platelet storage seems to be important for maintaining circulating levels of this potent immunoregulatory factor (Assoian et al. 1983). Whether TGFβ directly contributes to platelet–leukocyte interactions is not known, although limited data suggests that TGFβ may play a role in the prolongation of neutrophil survival sustained by activated platelets (Brunetti et al. 2000).

High mobility group box 1 (HMGB1) is a nuclear molecule highly conserved and abundantly expressed in human tissues. In contrast with most of the nuclear protein, HMGB1 transits continuously from the nucleus to the cytoplasm. In eukaryotic cells, HMGB1 is excreted from the cytoplasm in the presence of environmental inflammatory signals (Bianchi and Manfredi 2014; Lu et al. 2014). In the

extracellular milieu, HMGB1 acts as a potent inducer of the production of cytokines, the attraction of leukocytes, prompting, depending on the microenvironmental conditions, angiogenesis, and fibrosis. Although platelets are anucleated fragments derived from megakaryocytes, they contain substantial amounts of HMGB1 (Rouhiainen et al. 2000; Maugeri et al. 2012b, 2014a, c). In resting platelets, HMGB1 is mainly associated with cytoplasmic molecules, whereas upon activation HMGB1 is translocated to the platelet surface and released to the extracellular environment either as a soluble moiety or associated to the membrane of platelet-derived microparticles (Maugeri et al. 2012b, 2014c). Platelet-derived HMGB1 acts as a potent leukocyte agonist (Maugeri et al. 2014a, c; Vogel et al. 2015).

Complete platelet activation results in the release of the content of dense granules essentially ADP, ATP, and inorganic polyphosphate (Morrissey and Smith 2015). All these molecules modulate neutrophil and monocyte activity by acting through leukocyte purinergic receptors (Di Virgilio et al. 2001). Indeed, platelet-derived adenosine nucleotides enhance the oxygen respiratory burst in neutrophils which is mediated by (Ward et al. 1988) and upregulated by the β2 integrins on the neutrophil surface (Maugeri et al. 2009c).

Released from Neutrophils: Cathepsin G

Neutrophils contain four different granules (Table 2), primary (azurophilic granules), secondary (specific granules), tertiary (gelatinase granules), and quaternary (secretory granules) (Borregaard et al. 2007). Granule contents are usually sequentially released starting from secretory granules, followed by gelatinase granules, specific granules, and finally azurophilic granules (Pham 2006). At present four molecules released from activated neutrophils have been demonstrated to be able to modify the platelet response: proteinase 3 (Renesto et al. 1994, 1997), elastase (Selak 1992, 1994b; Si-Tahar et al. 1997; Renesto et al. 1993), cathepsin G (Selak et al. 1988; Selak 1994a; Maugeri et al. 1994; Cerletti et al. 1995; Molino et al. 1992, 1993, 1995; Si-Tahar et al. 1996), and pentraxin-3 (Maugeri et al. 2011b). While proteinase 3, elastase, and cathepsin G are potent platelets agonists, pentraxin-3 may inhibit P-selectin binding and reduce neutrophil responses. On the contrary pentraxin-3 may directly stimulate tissue factor expression in monocytes (Napoleone et al. 2002, 2004).

Prothrombotic and Proinflammatory Interactions

As described earlier, experimental models indicate that the recognition of P-selectin by PSGL-1 induces the leukocyte β2 integrin transactivation (Evangelista et al. 2007; Piccardoni et al. 2001, 2004), the release of neutrophil granule contents (Maugeri et al. 2005, 2007, 2009c, 2011b, 2012c), and the de novo synthesis and expression of preformed functional tissue factor in monocytes (Celi et al. 1994; Lorenzet et al. 1998; Cerletti et al. 2010) and neutrophils (Maugeri et al. 2006a, 2007; Maugeri and Manfredi 2015). Likewise, platelet–leukocyte heterotypic aggregates that characterize thrombotic, inflammatory, neoplastic, and autoimmune diseases were associated with the presence of tissue factor on the surface of neutrophils and monocytes (Maugeri and Manfredi 2015; Ritis et al. 2006; Ramirez et al. 2014; Baldini et al. 2014; Brambilla et al. 2008).

The adhesive interaction between P-selectin and PSGL1 also promotes arachidonic acid and eicosanoid exchange between platelets and neutrophils resulting in the transcellular synthesis of bioactive eicosanoids that modify

Table 2 Neutrophil granules content

Azurophilic granules (primary granules)	Specific granules (secondary granules)	Gelatinase granules (tertiary granules)	Secretory granules (quaternary granules)
Myeloperoxidase	Lactoferrin	Gelatinase	Complement receptor 1
Neutrophil elastase	Cathelicidin	Leukolysin	Complement receptor 3 (CD11bCD18)
Cathepsin G	Lysozyme	Lyzozyme	Formyl peptide receptor
Proteinase 3	Collagenase	Natural-resistance-associated-macrophage protein 1	CD14
Azurocidin	Complement receptor 3 (CD11bCD18)	Complement receptor 3 (CD11bCD18)	CD16
Bacterial permeability-increasing protein	Formyl peptide receptor	Acetyltransferase	Alkaline phosphatase
Defensins	Leukolysin		
	Cytochrome b558		
	Neutrophil gelatinase-associated lipacalin		
	Pentraxin-3		

platelet, leukocyte, and endothelial barrier function. Moreover, mainly through the release of HMGB-1, activated platelets promote NETs formation.

These prothrombotic and proinflammatory mechanisms, together with the capacity of neutrophils to downregulate some platelet functions, are described later.

Neutrophils Stimulate Platelets

The first evidence of the capacity of neutrophils to induce platelet aggregation through released cathepsin G was provided by Chignard (Selak et al. 1988). After this pioneering observation, the protease-activated receptor-4 (PAR-4) was suggested to be the main surface receptor transducing cathepsin-G-induced platelet activation (Sambrano et al. 2000).

Moreover, additional studies reported that cathepsin G also cleaves the main thrombin receptor (PAR-1) in human platelets, at the site Phe55-Trp56, thus deleting the tethered ligand domain and desensitizing platelets to thrombin (Molino et al. 1995), and cleaving GPIb (Pidard et al. 1994; Molino et al. 1993) and GPIX (Molino et al. 1993), thus reducing the ability of platelets to interact with vWF.

Through these mechanisms cathepsin G induces calcium mobilization and transduces signals by a pathway different from, but as effective as, that of thrombin (Molino et al. 1993). Platelet activation induced by cathepsin G, causes the rearrangement of the cytoskeleton (LaRosa et al. 1994a, b), and the release of both alpha and dense platelets granules (Si-Tahar et al. 1996), resulting in the expression of P-selectin on the platelet surface (Maugeri et al. 1994). Thus, when neutrophils release cathepsin G, neighboring platelets become activated and tightly adhere to neutrophils. Tight contact, in turn, is necessary for the transfer of metabolites during the transcellular metabolism of arachidonic acid and creates the microenvironment that protects cathepsin G and elastase (Evangelista et al. 1991, 1993) from the action of antiproteases present in plasma (Fig. 2). Elastase, also released from azurophilic granules upon neutrophil activation, acts in concert with cathepsin G (Selak 1992, 1994b; Si-Tahar et al. 1997; Renesto et al. 1993).

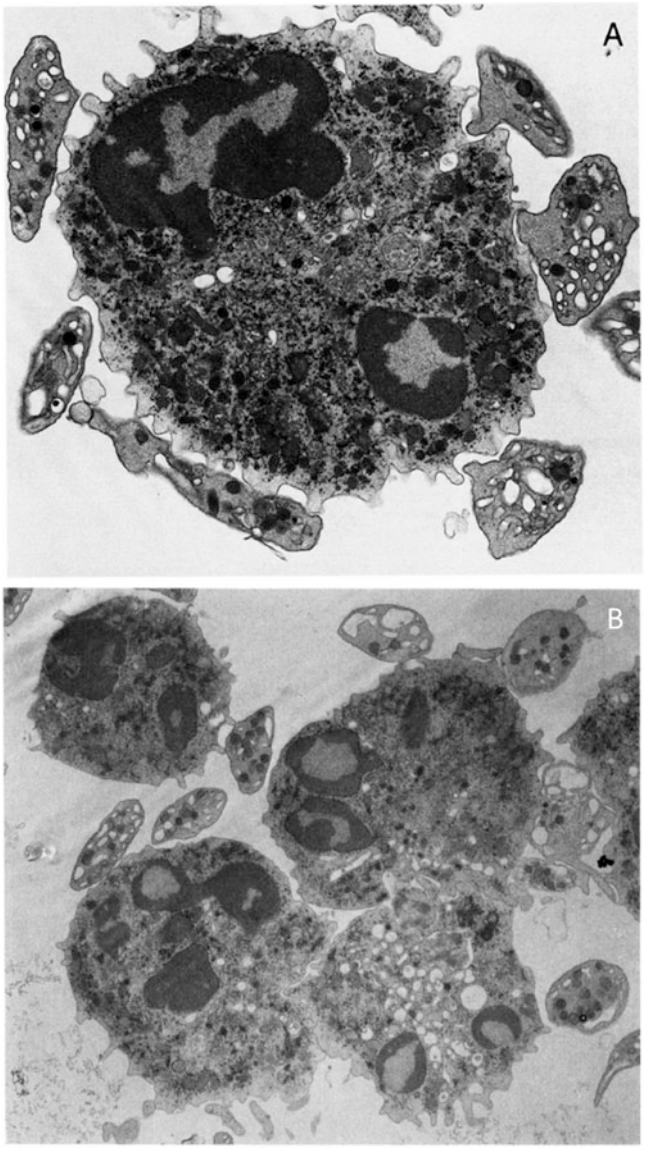

Fig. 2 The close contact between platelets and neutrophils creates a microenvironment that prevent the effect of plasma antiproteases. Electron microscopies of (**a**) platelets and neutrophils clumps and (**b**) platelet–neutrophils heterotypic aggregates. Panel **a**[113] and Panel **b**[112] were originally published in Evangelista et al., the American Society of Hematology

Platelets Stimulate Neutrophils

Mac-1 Transactivation

In neutrophils, the key molecular events which are induced by engagement of PSGL-1 by P-selectin that lead to β2 integrin activation include Src-family-kinase (SFK)-dependent phosphorylation of Nef-associated factor 1 (Naf-1), which is constitutively associated with the cytoplasmic domain of PSGL-1. The phosphorylated Naf-1 recruits phosphoinositide (PI)-3-OH kinase p85-p110δ, which then mediates Mac-1 activation. Mac-1 then initiates a SFK-mediated, outside-in signal that leads to phosphorylation of the proline-rich tyrosine kinase-2 (Pyk2), which stabilizes the integrin–ligand interaction and this forms neutrophil–platelet adhesion (Piccardoni et al. 2001, 2004; Evangelista et al. 2007) (Fig. 3).

Platelet-derived HMGB1, as well as platelet-derived microparticles bearing HMGB1, are able to upregulate and transactivate β2 integrins (Maugeri et al. 2014c). This

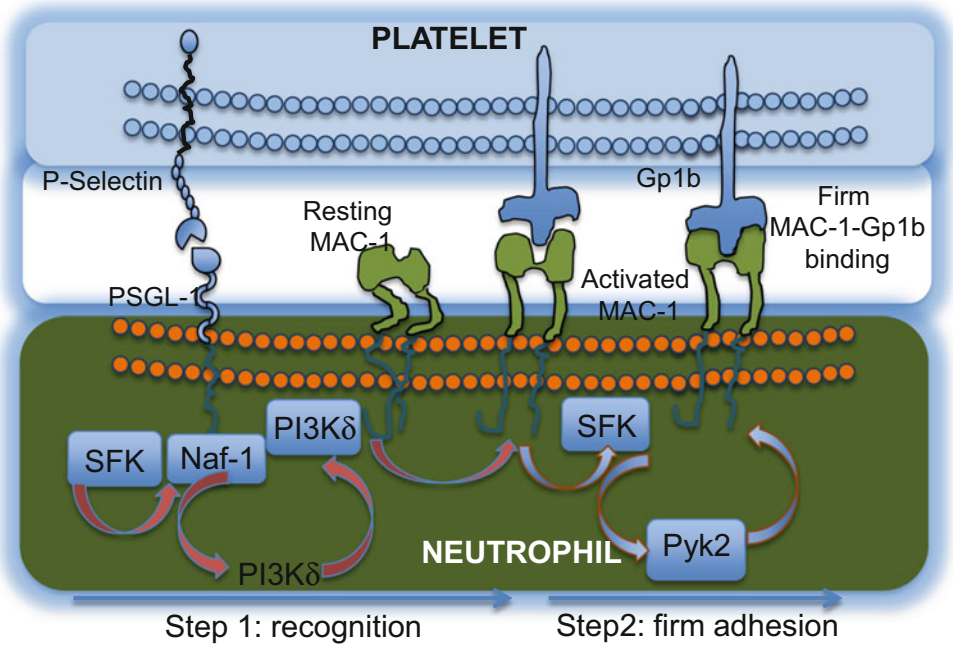

Fig. 3 Two-step model of platelet–neutrophil adhesion. P-selectin expression on the surface of activated platelets and its binding to neutrophil PSGL-1 triggers a dynamic cascade of signaling events and receptor–ligand pairs assembly that causes shear-resistant adhesion of neutrophils to platelets. In this model, the binding of P-selectin to PSGL-1 results in a SFK-dependent phosphorylation of Nef-associated factor 1 (Naf-1), which is constitutively associated to the cytoplasmic domain of PSGL-1. Phosphorylated Naf-1 recruits and activates phosphoinositide-3-kinase (PI3K)d which in turn mediates Mac-1 activation (Evangelista et al. 2007). Studies using a combination of pharmacological and genetic tools showed that the Src-family kinase (SFK) members Hck, Fgr, and Lyn also mediate signals downstream Mac-1 that are essential to stabilize Mac-1-ligand binding. These signals are required for Mac-1-dependent neutrophil recruitment by activated platelets along damaged arteries and for the subsequent development of intimal hyperplasia (Evangelista et al. 2007). In these studies, Pyk2 emerged as a novel potential regulator of αMβ2 downstream SFK. Thus, SFK may influence multiple steps in the signaling cascade leading to firm platelet–neutrophil interaction

pathway was shown to be regulated by the environmental conditions and specifically by the redox balance. Indeed HMGB1 oxidation, mediated by ROS formation by P-selectin-activated neutrophils, appears to amplify the ability of the moiety to functionally activate the leukocytes, possibly enforcing a self-sustaining positive feed-forward loop (Fig. 4).

Tissue Factor Expression

The first evidence of the involvement of P-selectin in thrombus formation was provided by Palabrica et al. (Palabrica et al. 1992) by using a dacron graft implanted within an artero-venous shunt in baboons. This study showed that P-selectin-mediated fibrin deposition in the growing thrombus mainly depended on the recruitment of granulocytes and of monocytes (Palabrica et al. 1992). In murine models, Furie et al. suggested that the process also requires the expression of tissue factor by the cells involved in the thrombus formation process (Furie and Furie 2008). In vitro observations reveal that P-selectin induces the de novo expression of procoagulant tissue factor in monocytes (Celi et al. 1994; Lorenzet et al. 1998) and neutrophils (Maugeri et al. 2006a; Maugeri and Manfredi 2015) and the release of the preformed molecule in a concentration-dependent manner.

Induction of Inflammatory Gene Expression

The interaction with platelets triggers delayed responses in leukocytes, which include gene expression and synthesis of inflammatory mediators. Delayed responses require the concerted actions of outside-in signaling that is transmitted by adhesive receptors (mainly PSGL-1 and β2-integrins) and of signals transduced by chemokine or cytokine receptors (Weyrich et al. 1995, 1996; Gawaz et al. 1998; Dixon et al. 2006). P-selectin and RANTES induce nuclear translocation of NF-kB, gene expression, and synthesis of MCP-1 and interleukin-8 (IL-8) in monocytes (Weyrich et al. 1996). The sustained adhesion of activated platelets induces cyclooxygenase (COX)-2 expression in monocytes through a mechanism that requires (i) the activation of PSGL1 due to platelet P-selectin (Weyrich et al. 1996) and (ii) the action of IL-1β released by activated platelets. Both agonists induce the activation and translocation of NF-kB and transcription of the COX-2 gene, and the translocation of COX-2 protein

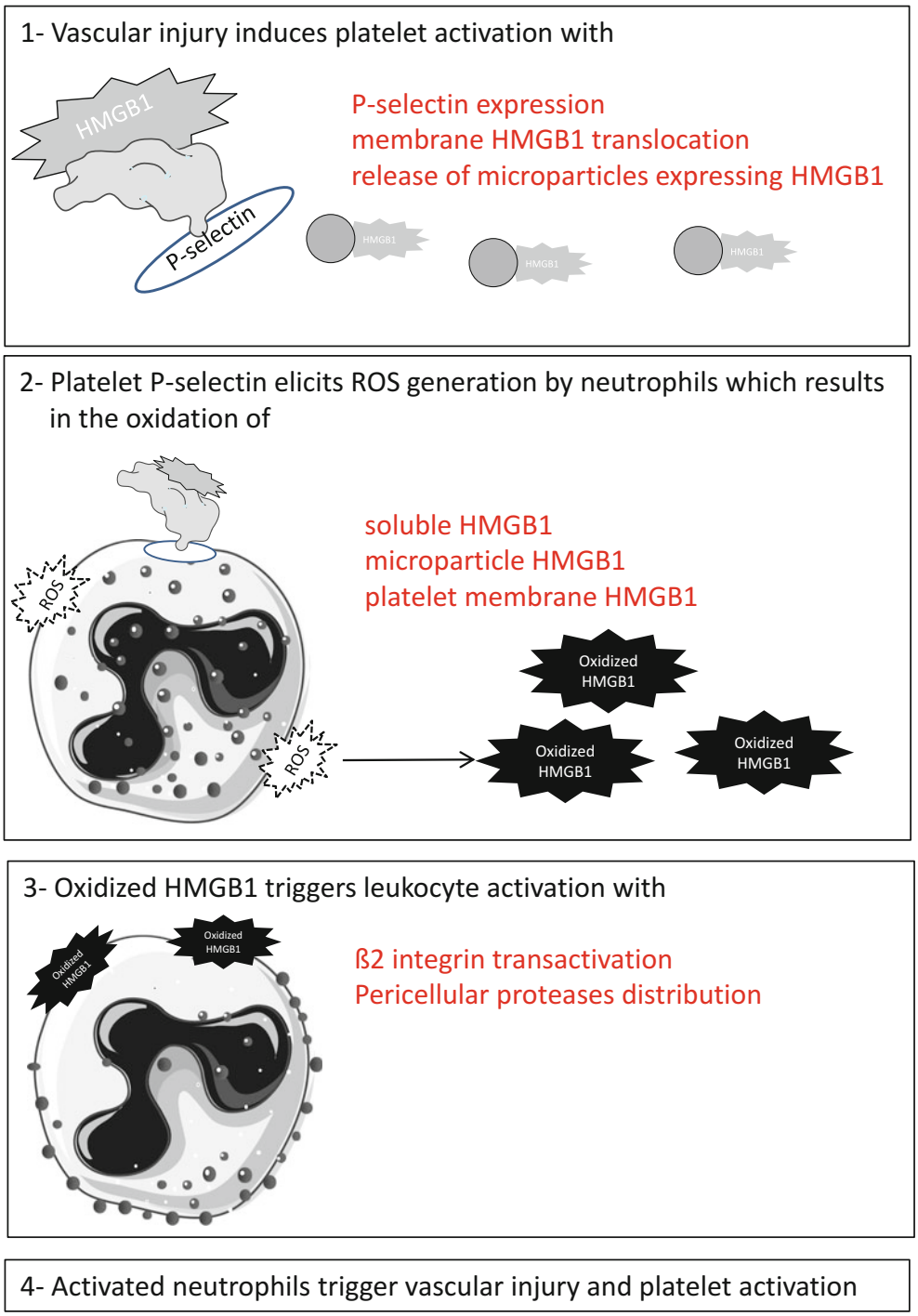

Fig. 4 Platelet–neutrophil cross-talk, role of HMGB1, and microparticles. Platelet P-selectin induces the neutrophil ROS formation and releases microparticles HMGB1^{+}. Oxidized HMGB1 promotes the pericellular distribution of granules and the transactivation of ß2 integrins

(Dixon et al. 2006). P-selectin/PSGL1 also promotes the tissue factor gene expression in monocytes (Di Santo et al. 2011) and neutrophils (Maugeri et al. 2006a).

Induction of ROS and Granule Secretion in Neutrophils

P-selectin expressed on activated platelets, as well as soluble P-selectin, induces the generation of ROS in intact neutrophils (Vanichakarn et al. 2008). The process of ROS generation requires leukocyte β2 integrins and platelet P-selectin and is sensitive to $P2Y_{12}$ antagonism, supporting a role for released ADP as discussed earlier (de Gaetano et al. 1999; Evangelista et al. 2005). The ROS produced by neutrophils can in turn enhance platelet activation and elicit the release of soluble CD40L (Vanichakarn et al. 2008). The generation of ROS by monocytes is also influenced by platelet-derived PF4/CXCL4, which serves as a potent inducer (Pervushina et al. 2004).

Transcellular Metabolism of Arachidonic Acid

Platelet–leukocyte interactions include cooperation in the arachidonic acid (AA) metabolism which has been defined as "transcellular metabolism" (Marcus 1986; Folco and Murphy 2006). Cytosolic phospholipase A2α translocates to the membrane and releases AA when cells are activated. AA becomes available to the enzymes involved in its metabolism: cyclooxygenases (COXs) and lypooxygenase-5 or -12 (5-LOx and 12-LOx, respectively) (Clark et al. 1995; Maclouf et al. 1998). 5-LOx and 15-LOx are expressed in neutrophils and monocytes while 12-LOx is expressed in platelets. 5-LOx is a cytosolic enzyme which translocates to the perinuclear membrane upon activation (Woods et al. 1993; Radmark et al. 2007). 5-LOx initially oxidizes free AA to 5-hydroperoxyeicosatetraenoic acid (5-HETE) and then in a second reaction dehydrates this intermediate to generate leukotriene (LT)A4 (Marcus et al. 1982) or lipoxins (LX) due to the action of 15-LOx (Serhan 1991). LTA4 is then further metabolized either to LTB4, by the action of cytosolic LTA4 hydrolase, or to LTC4 by LTC4 synthase, a nuclear envelop enzyme that conjugates LTA4 with glutathione essentially offered by platelets and endothelial cells (Maclouf et al. 1998). The final products of the 5-LOx pathway, LTB4 and peptido-LTs (LTC4, LTD4 and LTE4), play a fundamental role in inflammation because of their neutrophil activating effects (particularly in the case of LTB4) or ability to elicit smooth muscle cell contraction and edema (Peters-Golden and Henderson 2007). Intermediate compounds, mainly prostaglandin (PG)H2 and LTA4, are only partially metabolized intracellularly; a large proportion of intermediate metabolites exit from the cell to undergo nonenzymatic oxidation or to be taken up by adjacent cells (Borgeat and Samuelsson 1979).

At sites of inflammation where many cell types, platelets, and neutrophils, in particular, interact closely, the released AA and its metabolites can be exchanged. In this process, the donor cell provides intermediate metabolites that are transformed by the enzymatic machinery of the acceptor cell in a final active metabolite that none of the interacting cells would be able to produce alone (Marcus 1990). Platelets and neutrophils participate in this transcellular biosynthetic process in a bidirectional way. In fact activated platelets use AA released from adherent activated neutrophils to increase the production of TxA2. Furthermore, activated platelets provide free arachidonic acid to increase LTB4 production by the adherent neutrophils (Marcus et al. 1982; Palmantier and Borgeat 1991; Maugeri et al. 1992, 1994). Moreover, platelets utilize LTA4 from activated neutrophils to synthesize LTC4 (Maclouf and Murphy 1988; Maugeri et al. 1994). P-selectin-mediated membrane-to-membrane contacts facilitate the exchange of intermediate metabolites between neutrophils and platelets and promote transcellular synthesis of TxA2, LTB4, and LTC4 (Maugeri et al. 1992, 1994) (Fig. 5).

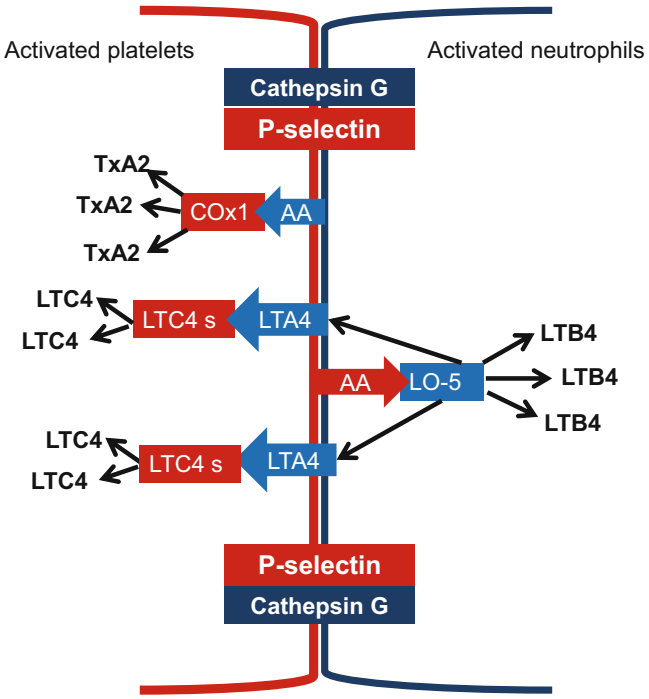

Fig. 5 Platelet–neutrophil cross-talk: transcellular metabolism of arachidonic acid

Platelet–leukocyte transcellular metabolism also leads to the generation of LX, an important class of anti-inflammatory mediators (Serhan et al. 2008), which have been implicated in the physiological termination of inflammatory responses. These metabolites arise from the combined action of 5-LOx and 12-LOx/15-LOx. Early studies demonstrated that coincubation of platelets and neutrophils in the presence of calcium ionophore generates LXA_4 as a consequence of the metabolic action of platelet 12-LOx on neutrophil-derived LTA_4 (Fiore and Serhan 1990; Serhan and Sheppard 1990; Romano and Serhan 1992).

Neutrophil Extracellular Traps

The discovery in 2004 of neutrophil extracellular traps (NETs) has modified the general vision of neutrophil biology and of key mechanisms of innate immunity. NETs are chromatin fibers decorated by neutrophil microbicide proteins, which are biologically active. NET generation thus represents a novel mechanism by which neutrophils kill microbes. More recently, the formation of NETs was observed in many sterile inflammatory disorders, including autoimmune (Radic and Marion 2013) and thrombotic diseases (Pfeiler et al. 2014) (Martinod and Wagner 2014).

NETs are apparently generated when neutrophils fail to engulf the pathogen because they are immobilized, adherent,

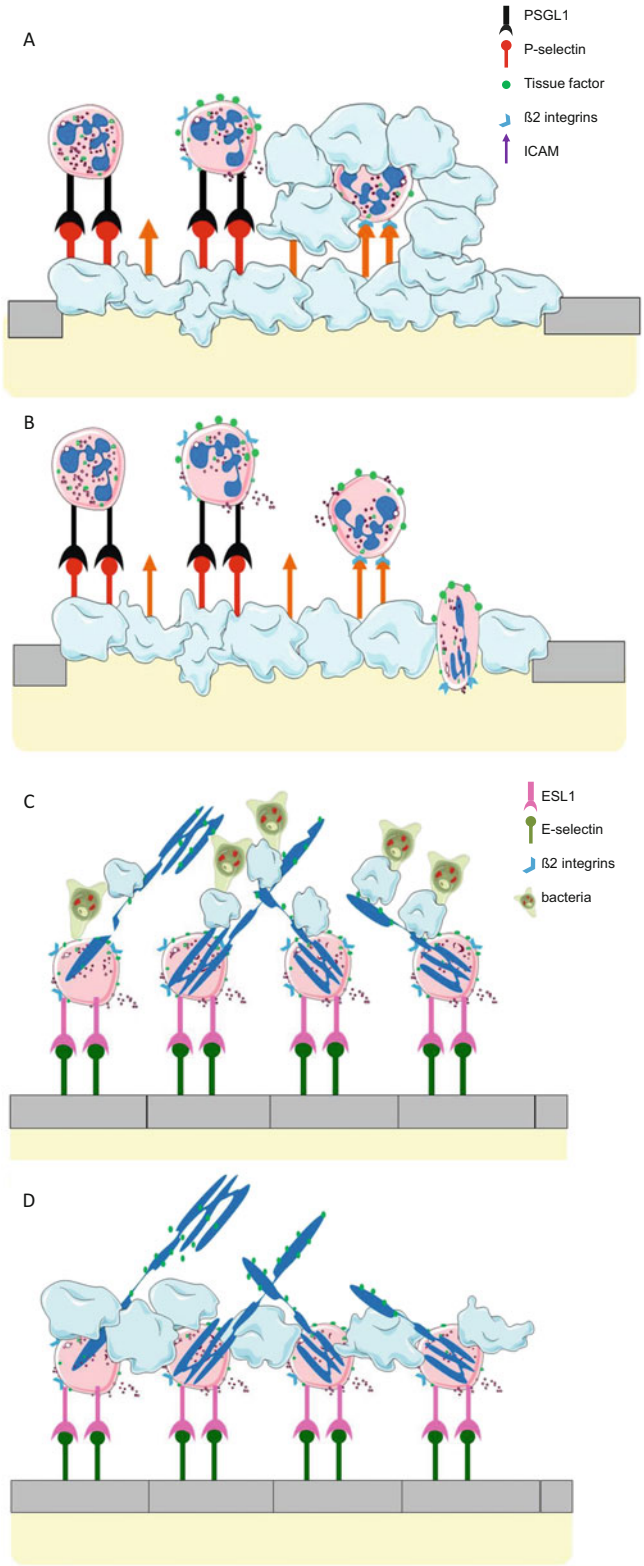

Fig. 6 Heterotypic interactions. Panel **a**: A modern vision of Bizzozero's observations: neutrophil PSGL1 recognizes P-selectin expressed by the activated platelets that adhere to denudated endothelium. The interaction induces neutrophil activation, tissue factor expression release of granular content, and the upregulation of ß2 integrins. The latter interacts with platelet integrins eliciting the formation of an heterotypic thrombi. Panel **b**: activated platelets, that have adhered to the subcellular matrix exposed as a consequence of vascular injury, express P-selectin and platelet integrins. The interaction with neutrophil PSGL1 induces neutrophil activation, tissue factor expression, release of granular content, and the asymmetrical (polarized) upregulation of ß2 integrins, resulting in the formation of heterotypic mural thrombi and/or the leukocyte transmigration. Panel **c**: model of platelet involvement in the NETs during infection. Activated endothelial cells express E-selectin, which is recognized by neutrophil ESL-1, resulting in the symmetrical (not polarized) upregulation of ß2 integrins. Platelets recognize bacteria and become activated and elicit the NETs formation increasing the capture of microbes. Panel **d**: Model of NETs formation in sterile conditions. Persistently activated endothelial cells express E-selectin, which is recognized by neutrophil ESL-1, resulting in the symmetrical (not polarized) upregulation of ß2 integrins. Leukocytes firmly adhere to endothelial cells. Activated platelets present HMGB1 and P-selectin to adherent neutrophils that induce the release of NETs that express tissue factor

or near to apoptosis (Manfredi et al. 2015; Yipp and Kubes 2013; Clark et al. 2007). Their generation depends on the fusion of primary granules to the nuclear membrane, the formation of myeloperoxidase– and elastase–DNA complexes (Papayannopoulos and Zychlinsky 2009; Papayannopoulos et al. 2010), and the citrullination of histones by peptidylarginine deiminase 4 (PAD4) (Leshner et al. 2012; Neeli et al. 2008).

As described earlier, neutrophils reach sites of injury and infection via a multistep process. The persistent endothelial activation induces the expression of E-selectin which is recognized by neutrophils through the constitutively expressed counter-receptor ESL-1 (Munoz-Caro et al. 2015; Hidalgo et al. 2009).

Intravascular generation of NETs may trigger thrombosis as the recognition of E-selectin, expressed on activated endothelial cells, by neutrophils through ESL-1 (Munoz-Caro et al. 2015; Hidalgo et al. 2009) (Fig. 6) initiates heterotypic interaction between neutrophils and endothelial cells resulting in a polarized distribution of integrins by which adherent neutrophils capture circulating platelets. In this multicellular context, signals of thrombo-inflammation and immune-thrombosis are thus integrated and may lead to the release of NETs (Maugeri et al. 2014a; Clark et al. 2007; von Bruhl et al. 2012).

The first evidence of the capacity of platelets to induce NETs was offered by Clark and collaborators (Clark et al.

2007) who demonstrated that platelets respond to gram-negative bacteria via activation of TLR4 receptors. In fact, in this model, neutrophils in the absence of adherent platelets do not respond to LPS (Clark et al. 2007; Maugeri et al. 2014a), but recognize platelets activated by gram-negative bacteria in the microcirculation. Neutrophil activation induced by LPS-stimulated platelets results in NET generation, thrombosis, and organ damage (Urban and Zychlinsky 2007). More recently, the development and use of surrogate animal models of venous thrombosis have demonstrated that monocytes, neutrophils, and platelets cooperate together to initiate and propagate venous thrombosis (von Bruhl et al. 2012), and that NETs are required for thrombus growth and stabilization. Other studies have shown that the infusion of NETs resulted in thromboembolism due to platelet activation, thrombin formation, and fibrin deposition (Fuchs et al. 2010).

A major mechanism by which platelets induce NETs formation is via HMGB1 (Maugeri et al. 2014a) (Fig. 7). The interaction of adherent neutrophils with activated platelets, or their challenge with supernatants obtained from activated platelets, induces NETs formation in static and flowing conditions, in the presence or absence of plasma and independently of the platelet agonist. All neutrophil responses associated to NETs formation induced by activated platelets or recombinant HMGB1 abated in the presence of competitive antagonists of HMGB1 or by using *Hmgb1*$^{-/-}$ platelets (Maugeri et al. 2014a; Vogel et al. 2015).

RAGE (Receptor for Advanced Glycation End products) is the receptor for HMGB1 that has been more clearly associated to NETs formation upon recognition of platelet-derived signals. The stimulation of RAGE by specific agonists (SA100 proteins, glycated albumin) induce NETs, and neutrophils from *Rage*$^{-/-}$ mice do not respond to activated platelets or to recombinant HMGB1 (Maugeri et al. 2014a; Vogel et al. 2015). In contrast, neutrophils from *MyD88*$^{-/-}$ mice (Maugeri et al. 2014a; Vogel et al. 2015) respond normally to platelets, confirming that neutrophil TLR signaling is not directly involved in platelet-induced NETs formation (Clark et al. 2007). Neutrophil exposed to recombinant HMGB1, as well as to activated platelets, activates the autophagy flux. As a consequence they maintain their mitochondrial potential, form autophagosomes and survive for longer time spans (Maugeri et al. 2014a). Recently, in vivo experiments by Vogel and collaborators (Vogel et al. 2015) further supported the contention that HMGB1-positive platelets are detectable along the NETs of coronary thrombi, and that deletion of platelet HMGB1 reduces/prevents thrombosis (Vogel et al. 2015).

Antithrombotic and Anti-inflammatory Interactions

Neutrophil Released Products That Downregulate Platelet Response

As described earlier, cathepsin G released from activated neutrophils may degrade platelet receptors by proteolysis. The degradation of GPIIbGPIIIa reduces platelet aggregation by preventing fibrinogen binding (Molino et al. 1993). Similarly proteolytic cleavage of GPIbα, GPIX and the (reversible) redistribution of GPV on the platelet surface induced by cathepsin G result in a reduction of the platelet capacity to respond to ristocetin and to bind von Willebrand factor, with a general impairment of their hemostatic and thrombotic capacity (Molino et al. 1993; LaRosa et al. 1994a). The pretreatment of platelets with cathepsin G also reduces their capacity to adhere to collagen and respond to thrombin and TxA2 (Kinlough-Rathbone et al. 1999). Finally, cathepsin G or neutrophil elastase cleaves PSGL1 indicating an autocrine mechanism for the downregulation of neutrophil adhesion-activated platelets (Gardiner et al. 2001).

Pentraxin 3

The presence of PTX3 associated to a substantial fraction of circulating platelets was observed 12 h after an acute myocardial infarction. In vitro observations confirm that PTX3 binds to activated platelets and reduces their ability to (1) form heterotypic aggregates with neutrophils and monocytes; (2) activate neutrophils, as evaluated by assessing the upregulation of Mac-1; (3) aggregate with other platelets; and (4) bind to fibrinogen. These results indicate that PTX3 released from activated neutrophils dampens platelet proinflammatory and prothrombotic actions (Baldini et al. 2012). PTX3 also binds to endothelial P-selectin and can attenuate neutrophil recruitment at sites of inflammation (Deban et al. 2010)

Clearance of Activated Platelets

Anionic phospholipids that represent a well-characterized phagocytic tag (or ‘eat me’ signal) are expressed together with P-selectin by activated platelets. The two signals may act in concert to promote capture of platelets by polarized clusters of neutrophil β2 integrins in vitro and in vivo. Neutrophils phagocytose activated platelets in circulation (Maugeri et al. 2009c, 2014b; Manfredi et al. 2010) and

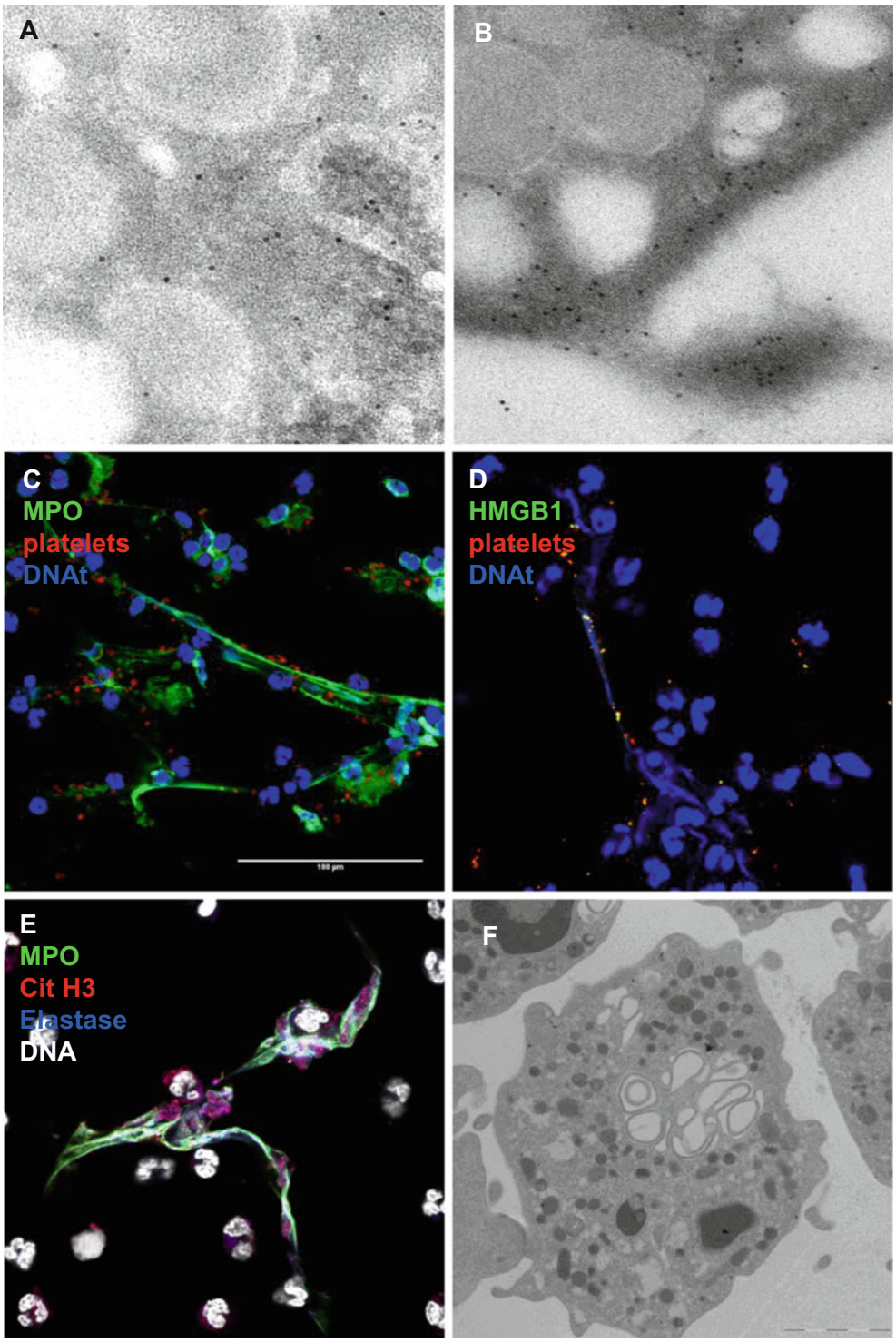

Fig. 7 Resting platelets content HMGB1 within cytoplasmic compartment. (**a**) HMGB1 expressed on activated platelets induce the NETs formation and neutrophil autophagy. Resting platelets content HMGB1 within cytoplasmic compartment (**a**) and is translocated to platelet surface when activated (**b**). (**c**) Adherent neutrophils challenged with autologous activated platelets (*red color*) generate NETs. NETs can be easily identified by the DNA lattices (*blue color*) decorated with MPO (*green color*). (**d**) Activated platelets (*red*) along NETs express HMGB1 (*green*). (**e**) Recombinant HMGB1 induce the NETs formation. NETs are characterized by the DNA (*white*) extrusion, the presence of citrullinated histone H3 (*red*), and the presence of MPO (*green*) and elastase (*blue*) along the DNA lattices. (**f**) Electron microscopy of neutrophils stimulated with HMGB1 illustrates the presence of autophagosomes. **Panels a–c,** and **f** were originally published by Maugeri et al. (2014a)

this clearance mechanism depends on the activation state of platelets, since their internalization requires the recognition of anionic phospholipids on platelets. This mechanism could play both an antithrombotic and anti-inflammatory action, since (i) it eliminates activated platelets that expose anionic phospholipids and tissue factor; (ii) render phagocytosing neutrophils into an unresponsive/anergic state that limits their reactivity to inflammatory stimuli. Accordingly, neutrophils from patients with Essential Thrombocythemia and Polycythemia Vera with thromboembolic events are apparently less effective at phagocytosing activated platelets in vivo (Maugeri et al. 2011a) (Fig. 8).

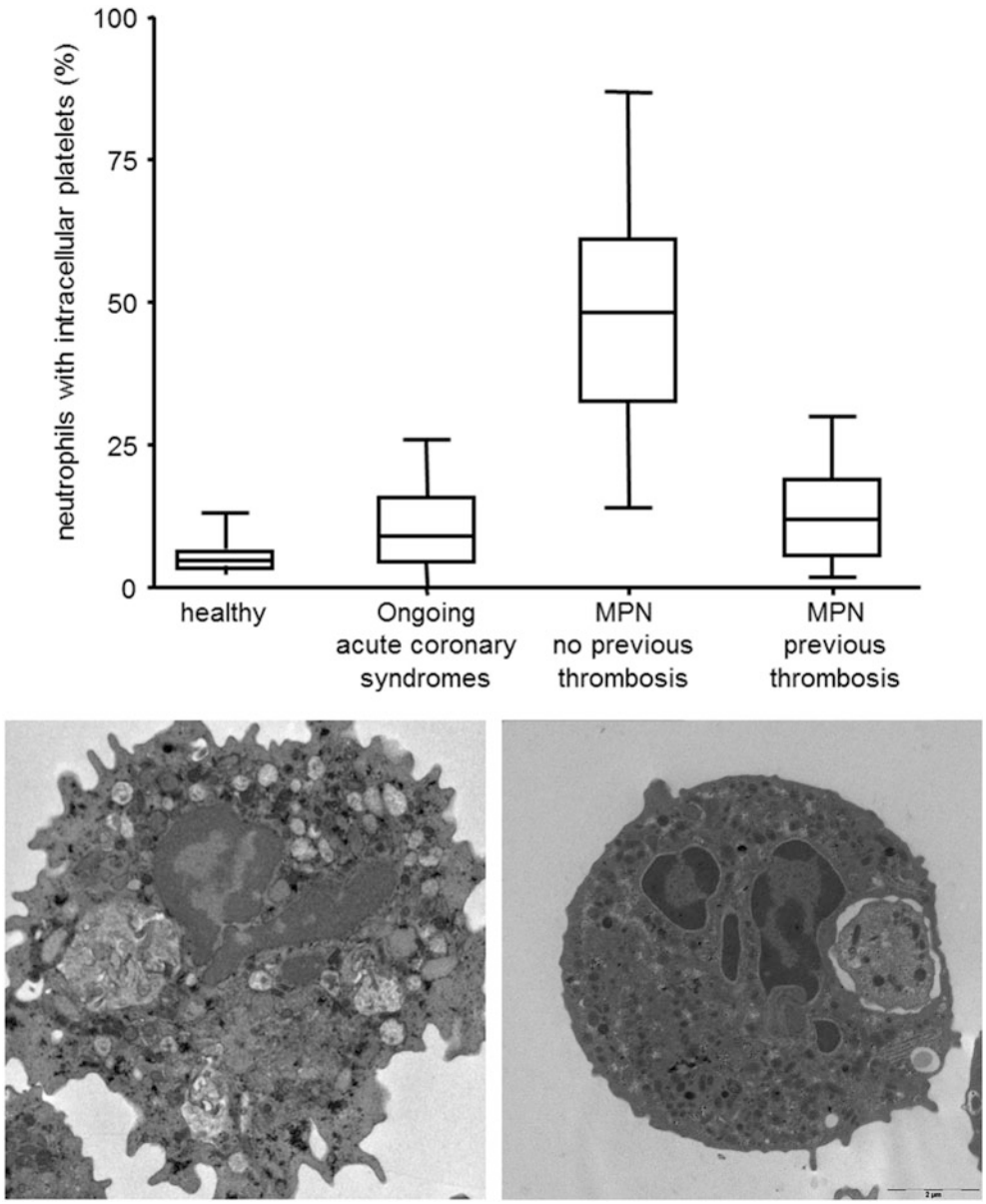

Fig. 8 Impaired clearance of activated platelets by neutrophils in patients with thromboembolic events. (**a**) The amount of neutrophils with intracellular platelets was determined in healthy donors, patients with acute myocardial infarction within 6 h from symptoms onset, patients with myeloproliferative neoplasms (MPN) with or without history of thrombotic events. Results of the plot were published by (Maugeri et al. 2009c, 2011a). (**b** and **c**) Representative electron microscopies illustrating the presence of intracellular platelets in neutrophils of patients with acute myocardial infarction and polycythemia vera, respectively, Images were originally published by (Maugeri et al. 2009c, 2011a)

CD39 Ecto-apyrase

Endothelial cells and leukocytes express the CD39 ecto-apyrase, which rapidly metabolizes ATP and ADP, thus reducing the nucleotide availability in the microenvironment and thus activation of purinergic receptors. As a consequence, the activity of CD39 also controls the migratory and inflammatory properties of neutrophils (Fung et al. 2009; Kukulski et al. 2011). Since platelets are a major source of agonistic nucleotides, these mechanisms may limit the activation of platelet and neutrophil activation in injured vessels and ischemic tissues (Fung et al. 2009; Hyman et al. 2009).

Pathological Features of Platelet and Leukocyte Interactions

Vascular Remodeling Responses

Platelets mediate leukocyte recruitment, primarily neutrophils and then monocytes, in the setting of endothelial injury, such as that occurs after angioplasty and stent deployment (Palmerini et al. 2002; Roque et al. 2000; Simon et al. 2000b; Smyth et al. 2001). Recruitment of inflammatory cells mediates the vascular response to injury

and neointimal formation (Roque et al. 2000; Simon et al. 2000b; Smyth et al. 2001). Platelet P-selectin is required for leukocyte recruitment and subsequent intimal hyperplasia (Wang et al. 2005a). P-selectin directly mediates platelet–leukocyte interactions and is required for the deposition of RANTES along the vessel wall, which also contributes to monocyte recruitment and the development of intimal hyperplasia (Schober et al. 2002). Moreover, in agreement with the concept that platelet-mediated leukocyte recruitment required coordinated actions of adhesive receptors, in vivo experiments in the mouse demonstrated that integrin αMβ2 and its main ligand on the platelet surface, GPIbα, are both essential for the recruitment of leukocytes at the site of vascular injury and for the subsequent development of intimal hyperplasia (Simon et al. 2000a, b; Wang et al. 2005b).

Atherosclerosis and Acute Coronary Syndromes

Studies in animal models support the contribution of activated platelets in the formation and progression of atherosclerotic plaques (Burger and Wagner 2003) through mechanisms that require expression of both P-selectin (Huo et al. 2003) and CD40L (Lievens et al. 2010), although there is conflicting evidence about the requirement for CD40L on bone marrow-derived cells in atherosclerosis (Bavendiek et al. 2005). Moreover, treatment with antibodies that inhibit platelet adhesive molecules mediating the interaction with leukocytes such as GPIbα (Massberg et al. 2002) or GPVI (Bultmann et al. 2010) and defects in platelet secretion (King et al. 2009) reduce atherosclerosis in mice (Schulz and Massberg 2017).

In hypercholesterolemic animals, activated platelets and platelet–leukocyte aggregates adhere to the endothelium at sites that are prone to plaque formation and deliver RANTES and PF-4. These signals in turn amplify the recruitment of monocytes and accelerate atherosclerosis (Huo et al. 2003). In humans, the increased fraction of circulating platelet–leukocyte heterotypic aggregates is considered as a hallmark of acute coronary syndromes (Maugeri et al. 2009c, 2011b, 2012c, 2014a; Furman et al. 2001; Michelson et al. 2001). Additionally, neutrophil infiltration at the culprit lesions in patients who have died following acute myocardial infarction suggests that platelet–neutrophil interactions occur at the site of ruptured plaques (Naruko et al. 2002).

The role of procoagulant tissue factor in coronary artery disease and acute coronary syndromes is well established. The exposure of tissue factor by the vessel wall at the site of endothelial injury plays a major role in the initiation phase, whereas blood-borne tissue factor would be involved more in the propagation phase of thrombus formation and coronary artery disease (Camera et al. 2012, 2015). Furthermore, the presence of tissue factor in circulating leukocytes and platelet–leukocyte heterotypic aggregates has been documented in patients with unstable angina and acute myocardial infarction (Brambilla et al. 2008).

The interaction of platelets and neutrophils in acute myocardial infarction seems to be unique in terms of the ability of activated platelets to induce neutrophil degranulation (Maugeri et al. 2011b, 2012c). Indeed, three subpopulations of circulating neutrophils have been described in patients during the very early phase of acute myocardial infarction: (1) newly formed neutrophils (expressing increased amounts of MPO), (2) neutrophils with low MPO content, and (3) neutrophils with complete MPO depletion. This three-modal pattern of MPO expression is specific of the first hours of an acute myocardial infarction and disappears 24–48 h after the onset of symptoms. MPO could not be detected in patients with unstable or chronic stable angina, and in patients with systemic inflammation due to either noninfectious (acute bone fracture), infectious (sepsis), or autoimmune diseases (small and large vessel systemic vasculitis, rheumatoid arthritis). In patients with an acute myocardial infarction, MPO depletion was associated with platelet activation, indicated by P-selectin expression, activation and transactivation of leukocyte β2-integrins, as well as the formation of platelet–neutrophil and –monocyte aggregates (Fig. 9). Likewise, the injection of activated platelets in mice produced P-selectin dependent complete MPO depletion in about 50 % of circulating neutrophils (Maugeri et al. 2012c).

The analysis of the composition of coronary thrombi obtained from patients with segment T elevation myocardial infarction reveals that neutrophils are the most abundant cell within the thrombi, having close engagement with activated platelets, the presence of HMGB1-positive platelets along the NETs (Maugeri et al. 2014a), and the TF associated to NETs structures in the culprit artery (Stakos et al. 2015) (Fig. 10).

Systemic Autoimmune Diseases

Vasculitides The clinical variability of vasculitides is characterized by a variety of pathophysiological causes that can be associated with established extravascular diseases, e.g., systemic autoimmune diseases, or occur as primary conditions and share clinical pathological features with other pathologies characterized by a prevalent vessel inflammation such as atherosclerosis, systemic sclerosis (SSc), and thrombotic microangiopathies (Ramirez et al.

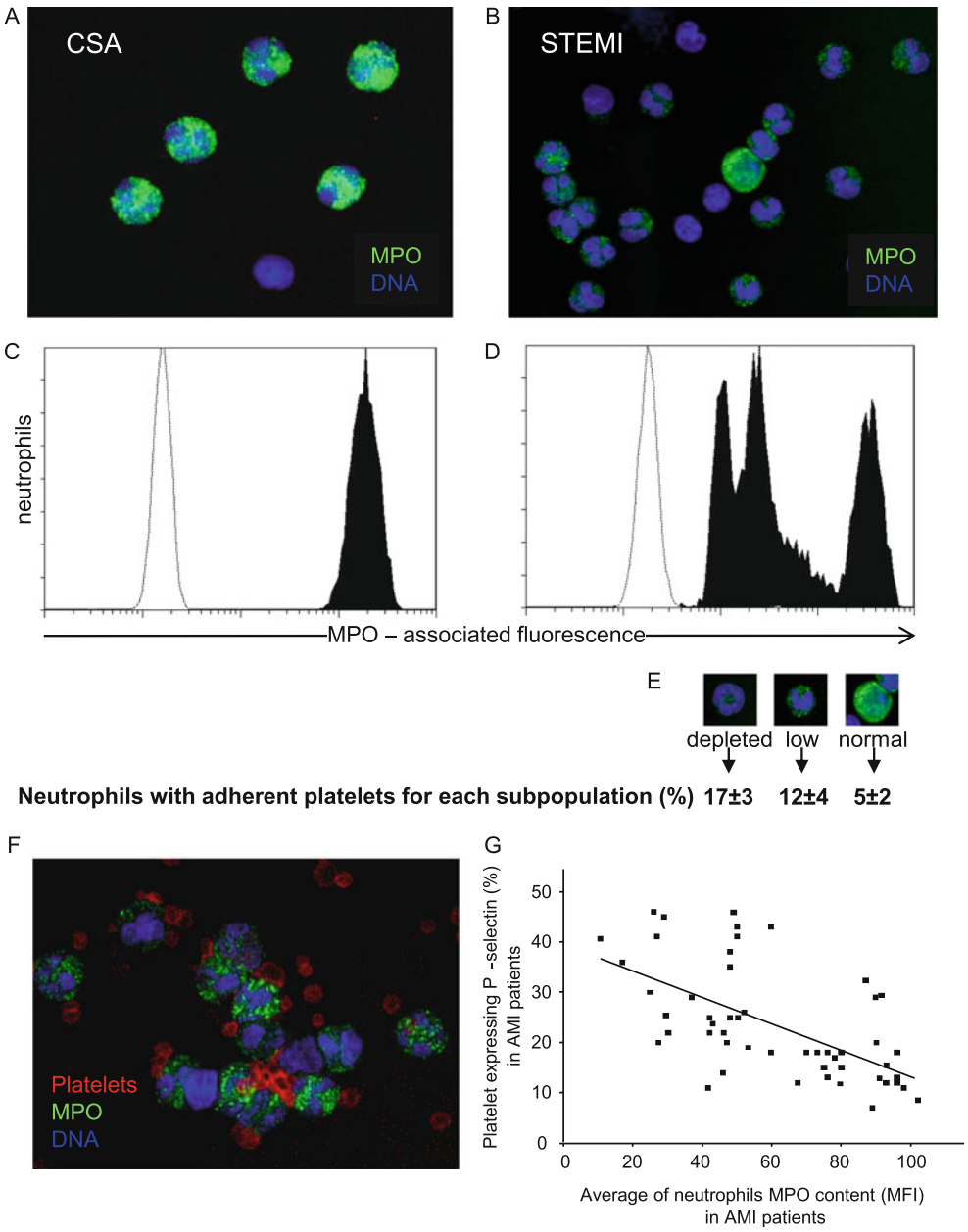

Fig. 9 Heterogeneity of myeloperoxidase content in circulating neutrophils from patients with acute myocardial infarction is associated to platelet adhesion and activation. Images were originally published by (Maugeri et al. 2012c)

2014; Baldini et al. 2014). Inflammation is, in most cases, self-sustaining with heterogeneous clinical manifestations, depending on the size of the vessels and the anatomical regions that are preferentially involved.

Large vessel vasculitides: An inflammatory process entirely developing inside the vessel wall characterizes large vessel vasculitis (Takayasu arteritis, giant cell arteritis or GCA) (Maugeri et al. 2009b; Grayson et al. 2012). GCA is the most common vasculitis affecting large vessels in the elderly. The onset is characterized by the arterial wall inflammation that leads to eventual occlusion of the vessel lumen and consequent ischemia. Thrombocytosis, activation of circulating platelets, platelet–leukocyte aggregates, and the expression of TF by circulating leukocytes are characteristics of GCA (Foroozan et al. 2002; Kale and Eggenberger 2010; Maugeri et al. 2009a, 2012c).

Small vessel vasculitides, also known as antineutrophil cytoplasmic antibodies (ANCA)-associated vasculitis (AAV), are characterized by the high prevalence of venous thromboembolism (Xu et al. 2015), platelet, and leukocyte activation (Maugeri et al. 2012c). Antibodies against neutrophil cytoplasmic antigens induce the generation of NETs, de novo synthesis of C-reactive protein (Xu et al. 2015; Peng et al. 2014; Radford et al. 2000). Circulating neutrophils in AAV express proteinase 3 on their surface which induces platelets activation (Peng et al. 2014).

In Behcet's disease, a rare, chronic multisystemic vasculitis, the occurrence of platelet–leukocyte interactions has

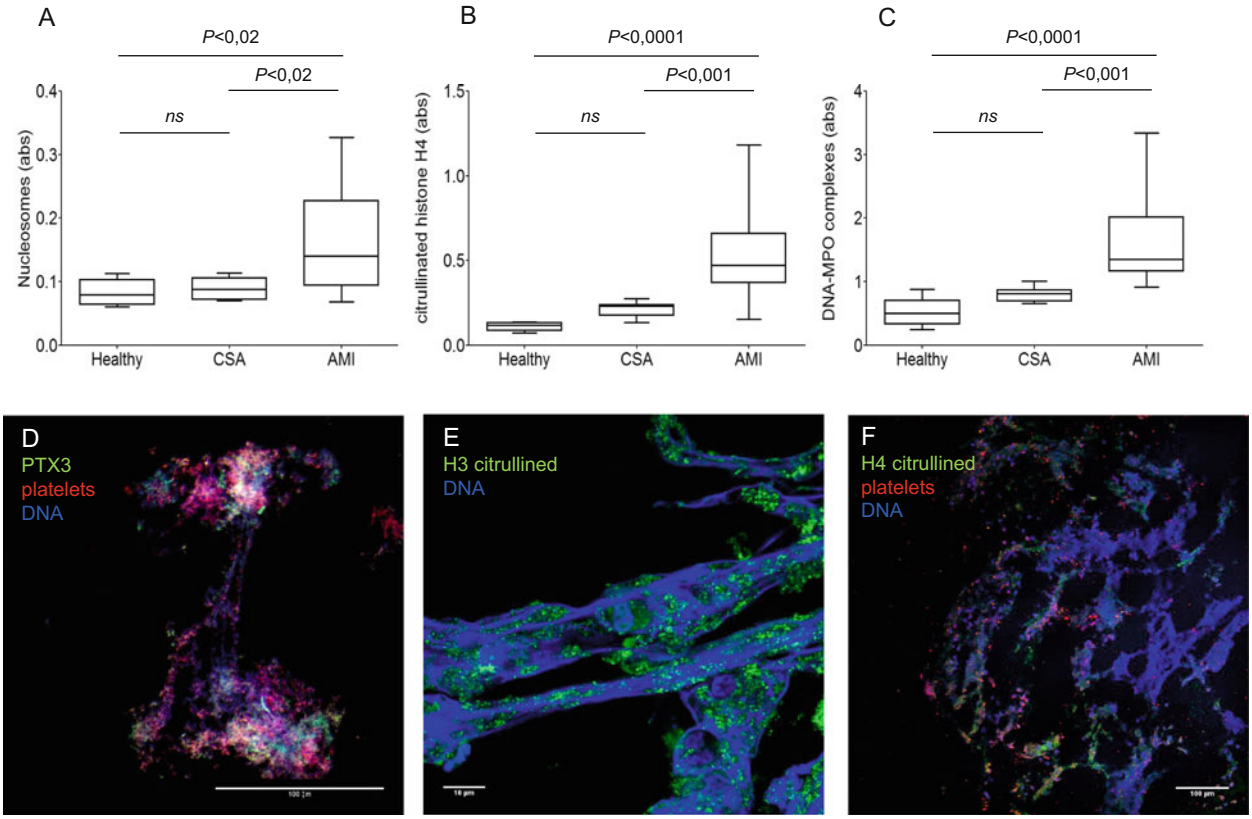

Fig. 10 NETs characterizes acute myocardial infarction. Increased plasmatic markers of NETs in patients with STEMI respect to healthy donors and patients with chronic stable angina. Results and images adapted from (Maugeri et al. 2014a)

been linked to vascular inflammation. In these patients, the presence of heterotypic aggregates, activated platelets, and platelet-derived microparticles appear to be associated with disease progression (Macey et al. 2011).

Rheumatoid Arthritis (RA) Platelets play a major role in RA since the presence of activated platelets and platelet-derived microparticles accumulate in the blood and the synovial fluid of patients with RA (Yaron and Djaldetti 1978; Knijff-Dutmer et al. 2002). Biologically active inflammatory microparticles are released as a consequence of platelet activation due to exposure to collagen and accumulate in the joints inducing the activation of fibroblastoid synoviocytes, which in turn secrete chemokines and cytokines that sustain synovitis and bone erosion (Boilard et al. 2010). These events might be important in the natural history of the disease, since the depletion of platelets reduces the severity of experimental murine arthritis (Boilard et al. 2010). Platelet-derived prostaglandins and serotonin contribute to the persistent inflammation and vascular permeability that characterizes RA synovial vessels (Boilard et al. 2012; Gasparyan et al. 2014). Thus, platelet activation is an early and possibly integral component of the vascular inflammation that is an hallmark of RA and may be a mechanism contributing to the increased cardiovascular risk in these patients. The platelet activation observed in RA extends to the formation of heterotypic aggregates with leukocytes, the consequent neutrophils and monocyte activation, and neutrophil degranulation (Maugeri et al. 2012c; Weissmann et al. 1982; Weissmann and Korchak 1984; Palmer et al. 1986; Bunescu et al. 2004; Manfredi et al. 2016).

Systemic Sclerosis (SSc) Oxidative stress is a critical player in the natural history of SSc, an inflammatory systemic disease associated with fibrosis, autoimmunity, and extensive vessel remodeling while the etiology of SSc is still unknown, platelets play a crucial role in SSc (Ramirez et al. 2012) since they show a unique biological behavior and represent a consistent circulating source of bioactive compounds that contribute to the development of many clinical features of the disease, such as formation of ROS, fibrosis, and impaired vascular tone (Ramirez et al. 2012).

At variance with the above-described mechanisms of platelet–leukocyte interaction observed in autoimmune-

inflammatory diseases, the effect of activated platelets on leukocytes seems to be exclusively dependent on platelet-derived microparticles bearing HMGB1 and on the oxidation state of HMGB1 (Maugeri et al. 2014c). Neutrophils and monocytes of patients with SSc show a dramatic decrease of PSGL1 and CD15 expression; in contrast, β2 integrins appear to be in a persistent activated state but without bound fibrinogen. Both, the loss of P-selectin receptors and the absence of fibrinogen on its receptors (β2 integrins) could be due to proteolytic cleavage mediated by released proteases (Maugeri et al. 2014c). The oxidation of HMGB1 amplifies its ability to activate neutrophils, as detected assessing the redistribution of primary granules molecules and the transactivation of the β2 integrins. In the context of platelet–leukocyte interactions, high amount of ROS can be generated by both activated platelets and by neutrophils triggered by P-selectin. In this context, oxidized extracellular HMGB1, soluble or associated to platelet membrane or to platelet-derived microparticles, further increased leukocyte activation. Leukocyte activation abated in the presence of inhibitors of HMGB1 or of catalase, which catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen. The redistribution of the content of primary granules and the activation of β2 integrins appear to be a hallmark of circulating neutrophils in SSc patients; in these patients, membrane HMGB1 is significantly higher in a subset of patients with pulmonary hypertension or with diffuse SSc. $HMGB1^{+}$ microparticles purified from SSc patients, but not $HMGB1^{-}$ microparticles purified from control subjects, activated in vitro healthy neutrophils and HMGB1 inhibitors reversed the effects of microparticles (Maugeri et al. 2014c) (Fig. 11).

Inflammatory Lung Diseases

A large variety of observational studies in patients and experimental studies in animal models have established a key role of platelet–leukocyte interactions in inflammatory lung disease. These studies emphasize the role of platelet–leukocyte interaction in the pathogenesis of clinical conditions characterized by acute lung injury (ALI) (Bozza et al. 2009) such as acute respiratory distress syndrome (ARDS) (Matthay and Zemans 2011) and transfusion-related lung injury (TRALI) (Caudrillier and Looney 2012).

Early studies showed the presence of platelets often associated with neutrophils and monocytes in lung vessels of histological samples from subjects with ALI/ARDS (Heffner et al. 1987) and suggested that platelets were required for complement-induced ALI in mice (Tvedten et al. 1985). More recently, taking advantage of novel pharmacological and genetic approaches, researchers have demonstrated that platelet–leukocyte interactions play a pathogenic role in ALI syndromes and identified mechanisms and mediators as potential targets for novel pharmacological strategies.

Using a murine model of acute lung injury, triggered by aspiration of hydrochloric acid (HCL), as a surrogate for human ALI/ARDS, Zarbock et al. (Zarbock et al. 2006) demonstrated that platelet depletion or administration of an anti-P-selectin antibody reduced neutrophil and protein accumulation in the airways, improved gas exchange, and increased animal survival. The use of chimeric mice indicated that platelet, but not endothelial, P-selectin was responsible for neutrophil recruitment and tissue injury (Zarbock et al. 2006). In vitro experiments identified thromboxane A2, which is released during platelet–neutrophil interactions, and signaling through thromboxane receptors, as a central mechanism stimulating ICAM-1 expression by endothelial cells, and contribute to neutrophil recruitment in the lungs (Zarbock et al. 2006). Overall these results were consistent with other studies in models of ALI indicating that treatment with anti-P-selectin antibody or genetic deletion of P-selectin protects animals from inflammatory lung damage (Kuebler 2006).

TRALI is the major cause of transfusion-related mortality, mainly mediated by passive infusion of human leucocyte antigen (HLA) and/or human neutrophil antigen (HNA) antibodies present in blood products. Although the pathophysiology of TRALI is still being defined, MHC antibody-based experimental TRALI models have revealed that platelets are recruited in the lung microvasculature and contribute to lung injury mainly through their interactions with neutrophils (Looney et al. 2009)

Using an elegant in vivo multichannel fluorescent imaging of the systemic circulation, Hidalgo et al. described the dynamics of heterotypic interactions of neutrophils with platelets in a two-event model of TRALI. In this model, the transfused antibody binds to endothelial MHC I antigen and mediates the capture of neutrophils via FcγR. In this context, E-selectin–ESL-1 engagement induces activation and polarization of the activated β_2 integrin Mac-1 at the neutrophil leading edge. In this position, Mac-1 mediates the capture of circulating platelets. The latter in turn triggers the generation of ROS leading to endothelial damage and microvascular occlusion (Hidalgo et al. 2009). In association with cell adhesion molecules, platelet-derived products, such as lysophosphatidylcholines (LysoPCs) which are released by aged PLTs during storage enhances neutrophil priming activity in vitro and triggers lung inflammation in healthy rats. By this mechanism, aged platelets contribute to pulmonary and systemic coagulopathy, in a "2-hit" TRALI model (Vlaar et al. 2010).

In a similar model, in vivo treatment with anti-P-selectin antibody reduced pulmonary edema, pulmonary and systemic inflammation, MPO activity, as well as pulmonary

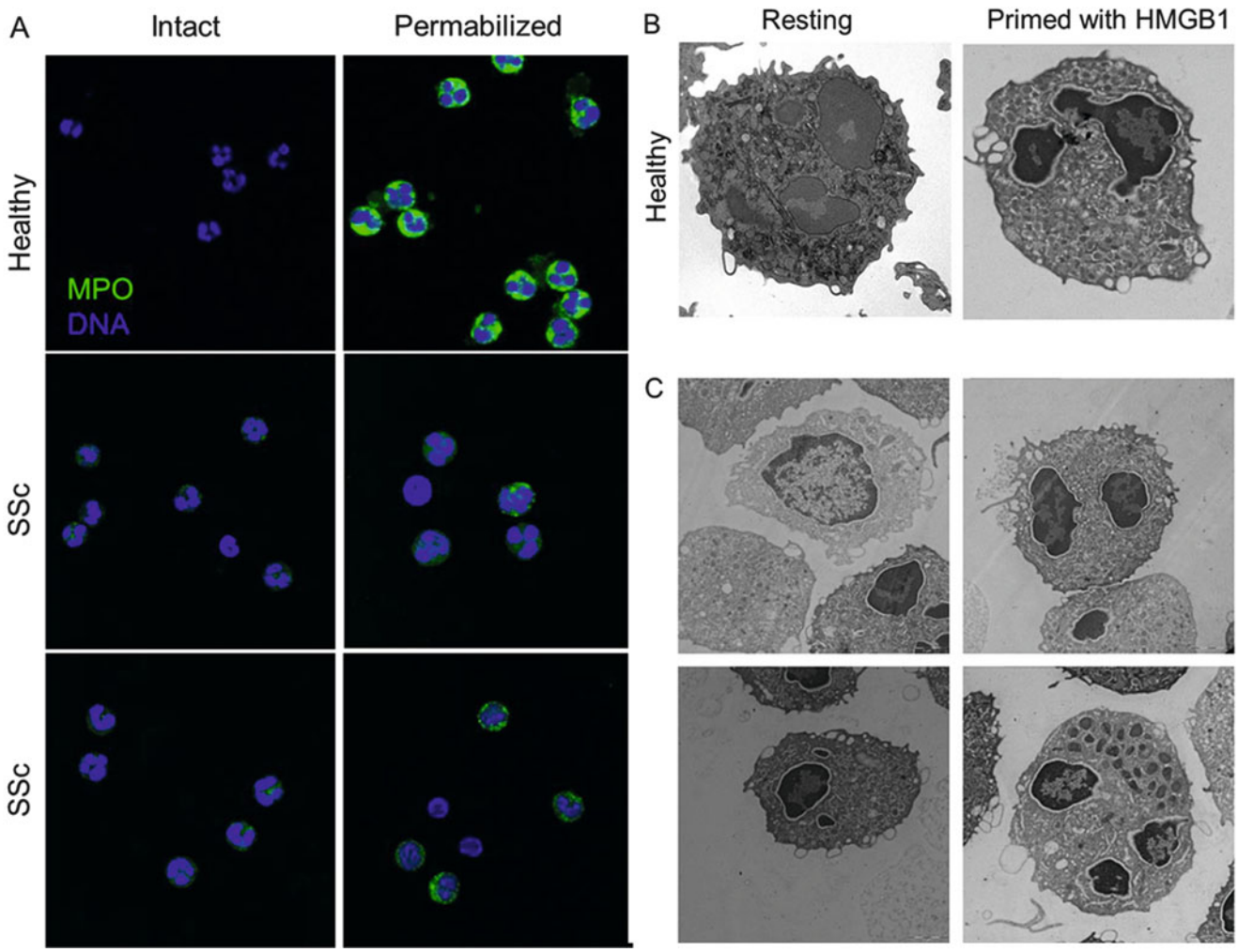

Fig. 11 MPO redistribution of circulating leukocytes of SSc patients. Representative images of neutrophils from the blood of patients with SSc and healthy controls. (**a**) The expression of MPO (*green*) in the blood neutrophils of patients with SSc and of matched controls has been analyzed by confocal microscopy before and after permeabilization of the plasma membrane, to allow the access of the mAb. Hoechst (*blue*) was used for counterstaining nuclei. MPO intracellular expression is substantially lower in SSc patients and appear to cluster at the plasma membrane of intact, nonpermeabilized neutrophils; (**b** and **c**) representative images by electron microscopy of neutrophils from a healthy control, in the absence or where indicated in the presence of recombinant HMGB1 (see Methods) (**b**) or of untreated neutrophils from four SSc patients (**c**). An extensive remodeling of intracellular granules, most of which acquire a pericellular distribution, characterizes SSc neutrophils and healthy neutrophils treated with HMGB1. Images were previously published by (Maugeri et al. 2014c)

and systemic coagulation, supporting the notion that P-selectin may play a key role in mediating TRALI (Tong et al. 2015).

Emerging evidence also indicates that NETs play important roles in inflammatory processes. Recent studies report that in a neutrophil- and platelet-dependent mouse model of ventilator-induced lung injury, NETs were found in the lung microvasculature, and circulating NET components were increased in the plasma. Pharmacological disruption of NETs, as well as the use elastase$^{-/-}$ mice, demonstrated that NETs directly influenced the severity of ALI (Rossaint et al. 2014).

Similarly to ALI, platelet–leukocyte interactions may play a role in allergic inflammation of the airways. In animal models of allergic asthma, platelet depletion reduced eosinophil and lymphocyte accumulation in the lungs after exposure of animals to specific allergens. Injection of wild-type, but not P-selectin-deficient platelets, restored leukocyte recruitment in the airways (Pitchford et al. 2005) (see also Momi et al. (2017)).

In addition to ALI and asthma, emerging hypotheses suggest that platelets may assist the recruitment of leukocytes into lung tissue in cystic fibrosis (CF) (O'Sullivan and Michelson 2006), a genetic disease in which mutations in

the gene of the CF transmembrane conductance regulator (CFTR) result in reduced secretion of Cl^- and HCO_3^- ions, thickened mucosal secretions, and chronic infections of the airways. An excessive and persistent accumulation of neutrophils, and tissue damage characterize the airways of patients with CF. These patients show increased levels of neutrophil–platelet and monocyte–platelet aggregates in their circulating blood, and increased Mac-1 expression on their neutrophils and monocytes (O'Sullivan and Michelson 2006; Mattoscio et al. 2010). Interestingly, in vitro blockade of CFTR in platelets and neutrophils from healthy subjects results in reduced generation of LX A4 by platelet–neutrophil coincubation and prolonged neutrophil survival (Mattoscio et al. 2010), suggesting that dysfunctional platelet–neutrophil interactions may contribute to the persistence of lung inflammation in CF. In animal models, LPS challenge of mice carrying the F508del (a common type of CFTR mutation) mutation induced more severe thrombocytopenia and had increased levels of plasma TXB2 (confirming the activating effect of LPS on platelets Maugeri et al. 2014a; Clark et al. 2007) that coincided with neutrophilic lung inflammation, relative to wild-type mice. Inhibition of platelets or depletion of neutrophils diminished LPS-induced lung inflammation in these F508del mice. In contrast, reconstitution of normal mice with F508del bone marrow or neutrophils resulted in a worse thrombocytopenia. Blockade of PSGL-1, in F508del mice reduced alveolar neutrophil transmigration after LPS challenge suggesting that F508del platelets and their interaction with neutrophils may contribute to the development of lung disease (Zhao et al. 2013).

Inflammatory Bowel Disease

Patients with inflammatory bowel disease (IBD) have an increased risk of microvascular thrombosis and thromboembolism (Yoshida and Granger 2009). Analysis of surrogate markers indicated the presence of thrombocytosis, platelet activation, and circulating platelet–leukocyte heterotypic aggregates in patients with IBD (Pamuk et al. 2006). This evidence can be recapitulated in the dextran sodium sulfate (DSS) and T-cell transfer models of murine colonic inflammation (Yan et al. 2013; Deban et al. 2008). The use of these animal models highlighted a key role for platelet–leukocyte–endothelial cell interactions in the pathogenesis of IBD (Gironella et al. 2002; Vowinkel et al. 2007). Intravital microscopy of colonic venules in mice subjected to experimental colitis has demonstrated that leukocytes that adhere to the vessel walls recruit the circulating platelets. The use of neutralizing monoclonal antibodies or the induction of colitis in P-selectin$^{-/-}$ mice indicated that P-selectin and PSGL-1 mediate the accumulation of platelets and leukocytes, and the extent of their accumulation correlates with disease severity (Gironella et al. 2002). In addition to P-selectin-PSGL-1 pair, CD40L appears to mediate platelet–leukocyte interaction in experimental colitis. Genetic deletion or pharmacological inhibition of CD40–CD40L pathway reduces platelet and leukocyte accumulation in the colonic microvasculature and attenuate the disease (Vowinkel et al. 2007). Thus, platelet and leukocyte recruitment in the microvasculature of the colon mucosa are codependent and might be responsible for microthrombosis, fibrin deposition, focal arteritis, and microinfarctions that have been observed in biopsies of patients with IBD.

Hypercoagulability and the prothrombotic state of the inflamed mucosal microvasculature are also sustained by increased expression of the procoagulant tissue factor. In a mouse model of colitis, a blockade of tissue factor using monoclonal antibodies prevented platelet and leukocyte recruitment and reduced tissue injury and microthrombosis (Anthoni et al. 2007).

In addition to contributing to thrombotic and inflammatory reactions at the intravascular side of the intestinal microcirculation, platelets migrate across the mucosal epithelium together with neutrophils. Interestingly, transmigrated platelets release large amounts of ATP, which is metabolized to adenosine by ectonucleotidases expressed on the apical surface of intestinal epithelial cells. Adenosine, in turn, induces chloride secretion and concomitant water movement into the intestinal lumen. In this way, transmigrated platelets can influence important functions at the luminal surface of the gastrointestinal epithelium.

Final Remarks

The molecular mechanisms and mediators of platelet–leukocyte cross-talk have been extensively investigated and there are consistent observations indicating that many of these can be considered potential targets for pharmacological intervention. For instance platelet-derived chemokines may be targeted to halt the progression of atherosclerosis, as demonstrated in hypercholesterolemic mice (Evangelista et al. 2007). Interference with P-selectin or PSGL-1, the main receptor pair mediating platelet–leukocyte adhesion successfully treated deep vein thrombosis in nonhuman primates (Myers et al. 2007). Finally, genetic and pharmacological targeting of the intracellular pathways that mediate Mac-1 activation has drawn attention to SFK- and PI(3)K-mediated pathways as novel targets for pharmacological control of thrombosis-related inflammation and vascular response to injury (Evangelista et al. 2007).

The important P-selectin/PSGL-1 axis could be disrupted by hydroxyurea. Indeed, hydroxyurea reduces thrombotic

complications in patients with myeloproliferative neoplasms and effectively prevents neutrophil tissue factor upregulation both in vitro and in vivo (Maugeri et al. 2006b, 2011a). A statistically significant reduction of the fraction of circulating neutrophils expressing tissue factor and of the neutrophil intracellular content was observed after hydroxyurea treatment of patients (Maugeri et al. 2006b, 2011a). Other P-selectin-dependent facets of neutrophil activation (neutrophil degranulation, upregulation of ß2 integrins) are also attenuated after treatment with hydroxyurea (Maugeri et al. 2006b, 2011a) reinforcing the likely mechanism of this drug.

In neutrophils, following binding to P-selectin, a phosphodiesterase type 4-sensitive cAMP pool negatively regulates SFK activity through the upregulation of COOH-terminal-Src-Kinase (CSK) and serves as an intrinsic "check" to inhibit platelet-mediated neutrophil recruitment at the site of endothelial damage. Thus, PDE4 inhibitors may represent a novel therapeutic strategy to counteract the detrimental consequences of platelet—neutrophil interactions in the context of vascular disease and atherothrombosis (Totani et al. 2014, 2015).

Heparin seems to be the most versatile drug to block the interactions between platelets and leukocytes. Independently of it anticoagulant effect, unfractionated heparin completely prevents the action of cathepsin G on platelets, including platelet aggregation, serotonin release, and TxA2 production. This effect seems to be dependent on the electrostatic interactions between the cationic cathepsin G and the anionic heparin that block the protease activity of cathepsin G (Evangelista et al. 1992; Ferrer-Lopez et al. 1992). Low molecular weight heparins (LMWH) also interfere with the formation of heterotypic aggregates of platelets and leukocytes, blocking the capacity of P-selectin to induce neutrophil degranulation and surface expression of TF (Maugeri et al. 2005, 2007). Furthermore, neutrophils treated with LMWH lose the capacity to respond to formylated peptide fMLP (Maugeri et al. 2005) in terms of degranulation and TF expression. LMWH also prevent platelet activation and P-selectin expression independently of the platelet stimuli used (Maugeri et al. 2007). In terms of NETs production, heparins prevent the neutrophil adhesion to activated endothelium since they interfere with P- and E-selectins (Wang et al. 2002), degrade NETs scaffolds (Fuchs et al. 2010, 2012), prevent the capacity of platelets to induce NETs (by blocking HMGB1) (Maugeri et al. 2014a), and inhibit MPO binding to DNA, thus restricting NETs formation (Parker et al. 2012). Heparin also prevents the effect of NETs on the fibrotic activity of differentiated lung fibroblasts (Chrysanthopoulou et al. 2014), influencing acute lung injury (Tong et al. 2014; Luan et al. 2014) and the binding of von Willebrand factor to neutrophils during thrombus formation (Grassle et al. 2014).

The studies cited in this chapter support the concept that platelet–leukocyte interactions represent a fundamental physiological process that bridges thrombosis and inflammation. Beyond atherosclerosis and thrombosis, a complex, bidirectional, functional cross-talk between platelets and leukocytes, mediated by specific adhesive molecules, soluble mediators, and membrane receptors plays a pivotal role in the pathogenesis of a variety of diseases, which supports the view that platelets are active components of the immune system and that activated leukocytes should be considered as thrombotic cells. In contrast, under healthy conditions platelet–leukocyte interactions seem to contribute to the resolution of inflammation and to the maintenance of vessel integrity (Maugeri et al. 2012a; Ramirez et al. 2014; Manfredi et al. 2010), and to the fine regulation of the amount of activated platelets present in the circulation (Maugeri et al. 2009c, 2011a; Manfredi et al. 2010).

Take-Home Message

Platelet–neutrophil interactions	
Prothrombotic and proinflammatory	Antithrombotic and anti-inflammatory
Neutrophils activate platelets by the release of cathepsin G and elastase	Cathepsin G and elastase proteolysis platelet glycoproteins and downregulate platelet function
Platelet activation results in the P-selectin and HMGB1 expression	Neutrophils phagocytose activated platelets by a mechanism dependent on P-selectin and Mac-1
Platelet P-selectin activate leukocytes and induce Mac activation, release of neutrophil granule content, the expression of tissue factor and ROS formation	Neutrophils with intracellular platelets are unable to react to inflammatory stimuli
HMGB1 expressed by activated platelets induce Mac-1 activation, pericellular granules distribution, and release of NETs	Pentraxin-3 released by activated neutrophils inhibits platelet function and the formation of heterotypic aggregates with leukocytes
NETs activate platelets	

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The Role of Platelets in Lymphangiogenesis and Neoangiogenesis

Katsue Suzuki-Inoue and Toshiaki Shirai

Abstract

During the developmental stages, when the primary lymph sac arises from the cardinal vein, podoplanin in lymphatic endothelial cells (LECs) activates platelets by binding to CLEC-2, which facilitates blood/lymphatic vessel separation. One of the mechanisms for this process is that granule contents from activated platelets inhibit the migration and proliferation of LECs, thereby facilitating separation of the vessels. Angiogenesis consists of several steps, including activation of endothelial cells (ECs), migration to the site of the vessel branch, proliferation, and formation of tube-like structures. Granule contents released from activated platelets, including VEGF and FGF, are required for EC proliferation. On the other hand, direct contact between platelets and ECs via platelet membrane proteins, including GPIbα and $\alpha_{IIb}\beta_3$, prompts angiogenesis by stabilizing developing vessels. Platelets also play a crucial role in maintaining the vascular integrity of high endothelial venules in lymph nodes and inflammatory vessels via the podoplanin receptor CLEC-2 and the collagen receptor glycoprotein VI. Platelets also regulate cerebrovascular patterning and integrity during development via CLEC-2 and podoplanin in neuroepithelial cells. Thus, platelets play a role, not only in thrombosis and hemostasis, but also in lymphangiogenesis, angiogenesis, and maintenance of vascular integrity.

The Role of Platelets in Blood/Lymphatic Vessel Separation During Development

It has long been recognized that platelets affect vascular endothelium stability, contributing to new vessel development (Gimbrone et al. 1969). However, only recently it has been shown that platelets play a role in lymphangiogenesis. Since 2010, several studies have revealed that platelets are involved in lymphangiogenesis, which was quite surprising. In this section, how platelets regulate proper lymphangiogenesis during development will be described starting with basic information about lymphangiogenesis.

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Development of the Lymphatic Vasculature

The lymphatic vessels arise after the cardiovascular system has been established. Lymphatic vessel development starts at around embryonic day (E) 9.5–10.5 in mice. At this stage, distinct subpopulations of vascular endothelial cells in the lateral parts of the cardinal veins commit to the lymphatic lineage (Fig. 1a). Then, endothelial cells committed to lymphatic endothelial cells (LECs) sprout laterally and migrate toward vascular endothelial growth factor (VEGF)-C to form primordial lymphatic vasculature, called lymph sacs (Fig. 1b) (reviewed in (Tammela and Alitalo 2010)). LECs begin to express the membrane protein podoplanin, which is a ligand for C-type lectin-like receptor 2 (CLEC-2) in platelets. The interaction between podoplanin in LECs and CLEC-2 in platelets helps to separate the blood and lymphatic vascular systems (Fig. 1c) (for details, see later). The

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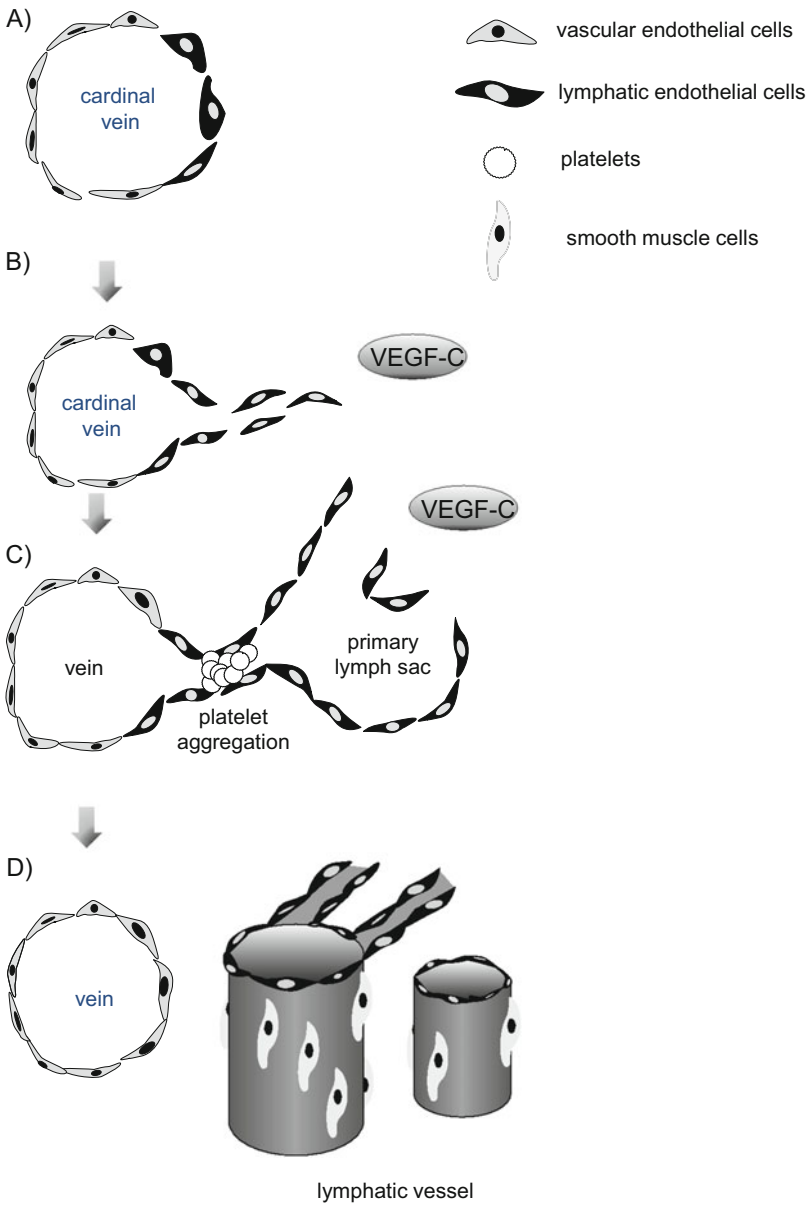

Fig. 1 Lymphatic vessel development. (**a**) Around embryonic day (E) 9.5–10.5 in mice, distinct subpopulations of vascular endothelial cells in the lateral parts of the cardinal veins commit to the lymphatic lineage. (**b**) Endothelial cells committed to lymphatic endothelial cells sprout laterally and migrate toward vascular endothelial growth factor (VEGF)-C to form primordial lymphatic vasculature, called lymph sacs. (**c**) Lymphatic endothelial cells begin to express the membrane protein podoplanin, which is a ligand for CLEC-2 in platelets. The interaction between podoplanin in LECs and CLEC-2 in platelets helps to separate the blood and lymphatic vascular systems (for details, see Fig. 2). (**d**) The peripheral lymphatic vessels are then generated by centrifugal sprouting of lymphatic vessels from the lymph sacs, followed by merging of the separate lymphatic capillary networks and remodeling and maturing of the primitive lymphatic capillary plexus

peripheral lymphatic vessels are then generated by centrifugal sprouting of lymphatic vessels from the lymph sacs, followed by merging of the separate lymphatic capillary networks, followed by remodeling and maturing of the primitive lymphatic capillary plexus (Fig. 1d).

C-Type Lectin-Like Receptor 2 (CLEC-2)

Identification of a C-type lectin family member, CLEC-2, on the surface of platelets revealed an unexpected role of platelets in lymphangiogenesis. CLEC-2 has been identified as a receptor for the platelet-activating snake venom rhodocytin, also known as aggretin (Suzuki-Inoue et al. 2006, 2011). CLEC-2 induces powerful platelet activation through the tyrosine kinase-dependent pathway, which closely resembles platelet activation mediated through the collagen receptor glycoprotein (GPVI/FcRγ-chain. CLEC-2 has a single YxxL motif in its cytoplasmic tail that resembles ITAM (tyrosine-based activation motif, YxxL-(X)10-12-YxxL), which has 2 YxxL motifs. ITAM is a signaling motif found in immune receptors such as the T-cell receptor and the platelet collagen receptor GPVI/FcR-γ-chain complex. Crosslinking of CLEC-2 or GPVI leads to tyrosine phosphorylation of hemi-ITAM or ITAM by the Src family kinases. This leads to binding of the tandem SH2 domain of the tyrosine kinase Syk to the phosphorylated (hemi-)ITAM. Subsequent activation of Syk initiates downstream signaling events that culminate in tyrosine phosphorylation of LAT and SLP-76 and activation of effecter

enzymes, including Btk and phospholipase Cγ2 (PLCγ2), resulting in platelet activation/aggregation (Suzuki-Inoue et al. 2006, 2011; Fuller et al. 2007; Navarro-Nunez et al. 2013; Watson et al. 2005).

Podoplanin

The membrane protein podoplanin has been identified as an internal ligand for CLEC-2 (Suzuki-Inoue et al. 2007; Christou et al. 2008). Podoplanin is a sialoglycoprotein expressed in certain types of tumor cells and various types of normal tissues, including LECs, type I alveolar cells, and kidney podocytes, after which podoplanin is named (reviewed in (Tsuruo and Fujita 2008)). Podoplanin is expressed in LECs, but not in vascular endothelial cells, and hence, it is used as a marker for LECs. Podoplanin expressed on the surface of tumor cells induces platelet aggregation by binding to CLEC-2 and facilitates hematogenous tumor metastasis (Tsuruo and Fujita 2008; Kato et al. 2008). Under physiological conditions, CLEC-2 in platelets cannot interact with podoplanin in LECs. However, as described earlier, the cluster of endothelial cells in the cardinal vein is committed to the lymphatic phenotype and sprout to form the primary lymphatic sacs during organ development (Fig. 1) (reviewed in (Tammela and Alitalo 2010)). At this stage, CLEC-2 in platelets can interact with podoplanin in LECs.

CLEC-2 in Platelets Facilitates Blood/Lymphatic Vessel Separation by Binding to Podoplanin in LECs

CLEC-2-deficient mice die at the embryonic/neonatal stages associated with disorganized and blood-filled lymphatic vessels and develop severe edema due to abnormal blood/lymphatic vessel separation (Bertozzi et al. 2010; Suzuki-Inoue et al. 2010). Platelet/megakaryocyte-specific CLEC-2-deficient mice also have blood-filled lymphatics (Finney et al. 2011; Osada et al. 2012), suggesting that CLEC-2 in platelets is required for blood/lymphatic vessel separation. Podoplanin deficiency and endothelial cell *O*-glycan deficiency also cause blood/lymphatic misconnections (Fu et al. 2008), and it has been shown that sialic acid on *O*-glycans of podoplanin is essential for binding to CLEC-2 (Suzuki-Inoue et al. 2007). These findings suggest that interaction between CLEC-2 in platelets and podoplanin in LECs is important for normal lymphatic vessel development.

Proposed Mechanisms for Blood/Lymphatic Vessel Separation by CLEC-2 in Platelets

The "Platelet Granule Contents" Theory

Mice deficient in the signaling molecules downstream of CLEC-2, Syk, SLP-76, and PLCγ2 have blood/lymphatic vessel misconnections (Ichise et al. 2009; Abtahian et al. 2003). Inhibition of platelet activation by treating pregnant wild-type mice with aspirin resulted in half of the embryos exhibiting blood/lymphatic misconnections (Uhrin et al. 2010). These findings suggest that platelet activation is required for blood/lymphatic vessel separation. Platelet activation results in granule release and platelet aggregation. Platelet granules contain many angiogenetic factors and growth factors, as well as extracellular matrix, implying that these factors could contribute to blood/lymphatic separation. Alternatively, platelet aggregates accumulate at the separation zone of lymph sacs and cardinal veins, which helps the physical process of separation. In fact, Uhrin et al. and Bertozzi et al. reported that platelet aggregates accumulate at the separation zone of podoplanin-positive lymph sacs and cardinal veins in wild-type embryos, but not in podoplanin-deficient or SLP-76-deficient embryos (Uhrin et al. 2010; Bertozzi et al. 2010). However, mice deficient in integrin αIIbβ3, which is necessary for platelet aggregation, but not for granule release, do not show the nonseparation phenotype (Bertozzi et al. 2010), suggesting a role of granule release. Moreover, supernatants from activated platelets and their main component, TGF-β, inhibit the migration and proliferation of LECs (Osada et al. 2012). In addition to TGF-β, platelets contain some proteins that reportedly inhibit the proliferation or migration of LECs (Table 1) (Oka et al. 2008; Shao and Xie 2005; Shao and Chi 2005). Osada et al. proposed the following mechanisms (Fig. 2a): during the developmental stages, primary lymph sacs derive from the cardinal vein. In the connection between the lymph sac and vein, podoplanin in LECs activates platelets by binding to CLEC-2. The released TGF-β family members inhibit LEC migration, proliferation, and tube formation. This results in the elimination of LECs close to activated platelets, which facilitates blood/lymphatic vessel separation. However, patients who lack platelet dense or α-granules do not exhibit defective blood/lymphatic vessel separation (Michelson 2012). Further studies are therefore necessary to clarify this mechanism.

The "CLEC-2-Mediated Podoplanin Clustering" Theory

Pollitt et al. proposed another mechanism (Pollitt et al. 2014) (Fig. 2b) in which CLEC-2 forms a central cluster upon engagement with podoplanin. Clustering of CLEC-2, in

Table 1 Pro/antiangiogenic factors and antilymphangiogenic factors stored in platelets

Angiogenesis activators (soluble factors)	Angiogenesis inhibitors
Vascular endothelial growth factor (VEGF) (Brill et al. 2005; Gerhardt 2008)	Angiostatin (Jurasz et al. 2003)
Platelet-derived growth factor (PDGF) (Heldin 1997)	Endostatin (Battinelli et al. 2011)
Fibroblast growth factor (FGF) (Brunner et al. 1993)	Thrombospondin-1 (TSP1) (Zaslavsky et al. 2010)
Epidermal growth factor (EGF) (Viloria-Petit et al. 2001; Lee et al. 2004; Nakamura et al. 1989)	Platelet factor 4 (Brill et al. 2004)
Insulin-like growth factor (IGF) (Shigematsu et al. 1999; Nicosia et al. 1994)	
Angiopoietin (Nicosia et al. 1994)	
Stromal cell-derived factor-1 (SDF-1) (Jin et al. 2006; Stellos and Gawaz 2007)	Lymphangiogenesis inhibitors
CD40 ligand (CD154) (Rutella et al. 2011)	Transforming growth factor β (TGF-β) (Osada et al. 2012; Oka et al. 2008)
Matrix metalloproteinases (MMPs): MMP-1, MMP-2, MMP-9 (Weyrich et al. 2003)	Platelet factor 4 (Shao and Xie 2005)
Sphingosine-1-phosphate (S1P) (Yatomi et al. 2000; English et al. 2000, 2001)	Endostatin (Shao and Xie 2005)
Heparinase (Brill et al. 2004; Vlodavsky et al. 1992)	Angiostatin (Shao and Chi 2005)

turn, induces clustering of podoplanin. CLEC-2 clustering is dependent on Syk and Src kinases, both of which are signaling molecules downstream of CLEC-2 in platelets. Clustering of podoplanin leads to functional effects including inhibition of migration in LECs by podoplanin-mediated signaling though ezrin/radixin/moesin family proteins and regulation of the actin cytoskeleton. In support of this theory, Osada et al. also reported that recombinant CLEC-2 alone inhibits LEC migration without platelet releasates, although it does not inhibit LEC proliferation (Osada et al. 2012). These findings account for the similar lymphatic phenotype of CLEC-2- and Syk-deficient mice.

The "Prevention of Backflow at the Lymph-Venous (LV) Junction" Theory

The vascular networks connect at the LV junction, where lymph drains into the blood and an LV valve (LVV) prevents the backflow of blood into lymphatic vessels. Hess et al. reported that loss of CLEC-2 resulted in backfilling of the lymphatic network with blood from the thoracic duct in both neonatal and mature mice, even when LVVs are intact (Hess et al. 2014). Interaction between platelet CLEC-2 and podoplanin at LVVs results in the formation of platelet thrombi. Fibrin-containing platelet thrombi were observed at the LVV and in the terminal thoracic duct in wild-type mice but not CLEC-2 deficient mice. It is plausible therefore that hemostasis via CLEC-2 functions with the LVV to safeguard the lymphatic vascular network throughout life (Fig. 2c).

These three mechanisms may not be exclusive of each other, and all three mechanisms may exist in vivo.

The Role of Platelets in Angiogenesis

For successful angiogenesis, endothelial cells need to complete several steps: activation, migration to the site of the vessel branch, proliferation, and formation of tube-like structures that are used in the new vessels (Jain 2003; Klement et al. 2013). Soluble factors, including VEGF and FGF, released from activated platelets, but not contact between platelets and endothelial cells, are required for the proliferation of endothelial cells (Pintucci et al. 2002). On the other hand, whole platelets, but not releasates from platelets, can induce tube formation by endothelial cells (Pipili-Synetos et al. 1998), suggesting that direct contact between platelets and endothelial cells is required for this process. Thus, both soluble factors and direct contact via membrane proteins on the surface of both types of cells are required for the different steps of angiogenesis.

Contributions of Soluble Factors Stored in Platelets to Angiogenesis

Platelets contain a number of angiogenesis activators and inhibitors (Table 1) (Klement et al. 2013). These factors are released upon platelet activation and delivered to the site of neoangiogenesis directly or as intraparticle cytokines contained in platelet-derived microparticles (Brill et al. 2005). Brill et al. reported the proangiogenic effects of platelet-derived microparticles in vitro (using the rat aortic ring model or the cell invasion test) and in vivo (using agarose bead transplantation or artificial cardiac ischemia in Sabra rats). They proved that these effects are mediated by intraparticle cytokines, i.e., VEGF, βFGF, and PDGF, using appropriate blocking antibodies (Brill et al. 2005). It has previously been reported that pro- and antiangiogenic proteins are separated in distinct subpopulations of α-granules in platelets and megakaryocytes (Italiano et al. 2008; Chatterjee et al. 2011). Human platelets express two types of thrombin receptors, proteinase-activated receptor 1 (PAR1, high affinity) and PAR4 (low affinity). Treatment of human platelets with a selective PAR4 agonist results in the release of granules containing antiangiogenic protein,

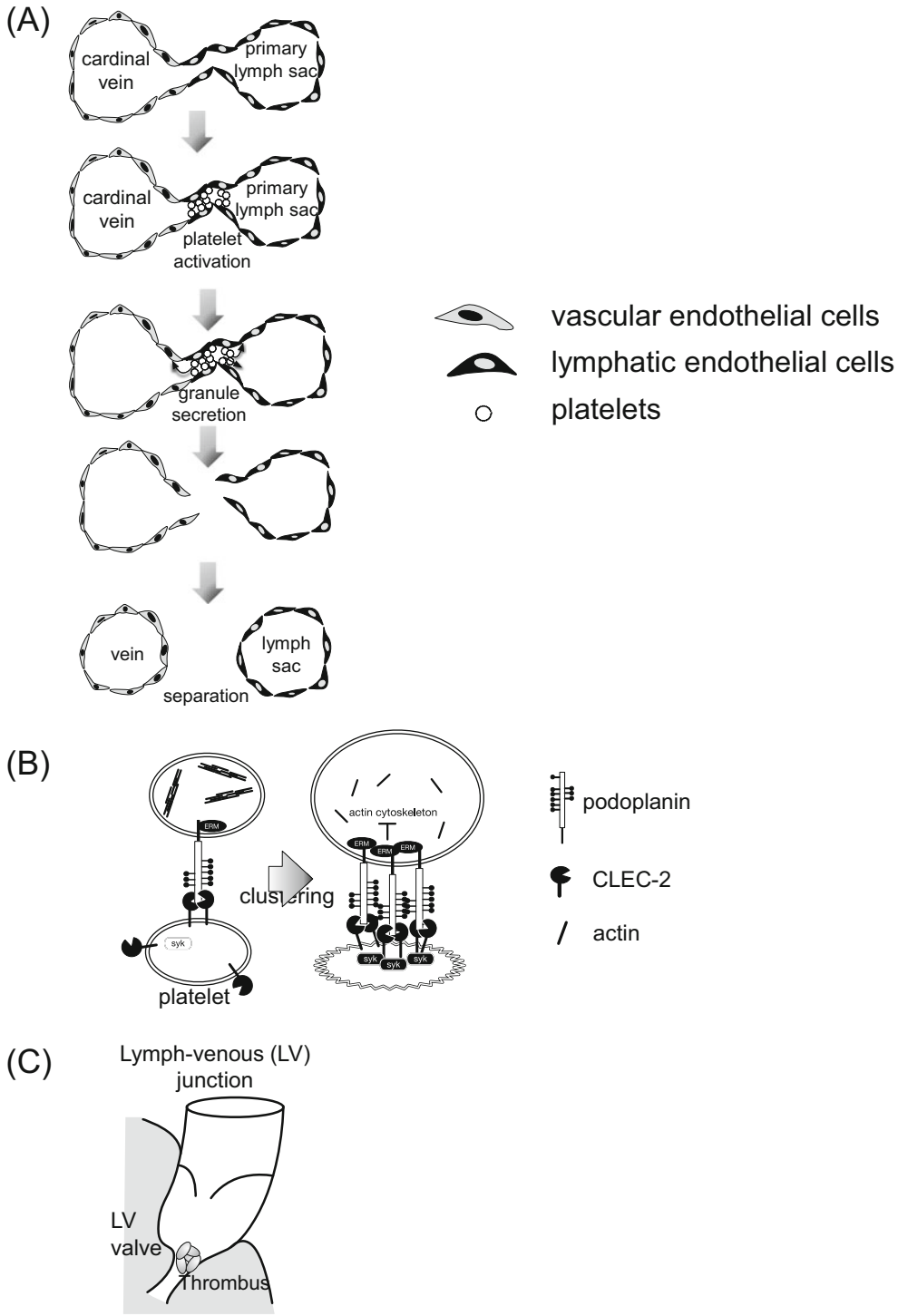

Fig. 2 Proposed mechanisms of blood/lymphatic vessel separation by platelet CLEC-2. (**a**) The "platelet granule contents" theory: during the developmental stages, primary lymph sacs arise from the cardinal vein. In the connection between the lymph sac and vein, podoplanin in lymphatic endothelial cells (LECs) activates platelets by binding to CLEC-2. The released TGF-β family members inhibit LEC migration, proliferation, and tube formation. This results in the elimination of LECs close to activated platelets, which facilitates blood/lymphatic vessel separation. (**b**) The "CLEC-2-mediated podoplanin clustering" theory: CLEC-2 forms a central cluster upon engagement with podoplanin. Clustering of CLEC-2, in turn, induces clustering of podoplanin. CLEC-2 clustering is dependent on Syk and Src kinases, both of which are signaling molecules downstream of CLEC-2 in platelets. Clustering of podoplanin leads to functional effects including inhibition of migration in the LECs by podoplanin-mediated signaling though ezrin/radixin/moesin family proteins and regulation of the actin cytoskeleton. (**c**) The "prevention of backflow at the lymph-venous (LV) junction" theory: the vascular networks connect at the LV junction, where lymph drains into the blood and an LV valve (LVV) prevents the backflow of blood into lymphatic vessels. Interaction between platelet CLEC-2 and podoplanin at LVVs results in the formation of platelet thrombi at LVVs, which prevents backfilling of the lymphatic network with blood from the thoracic duct

but not granules containing proangiogenic protein, whereas the selective PAR1 agonist liberated proangiogenic granules, but not antiangiogenic granules (Italiano et al. 2008; Chatterjee et al. 2011; Ma et al. 2005). In the case of early injury, when there is only a small amount of thrombin, the high-affinity thrombin receptor (PAR1) is engaged, and proangiogenic proteins are released. In later stages of injury, when a high thrombin state occurs because the majority of the clot is crosslinked by factor XIII, the low-affinity thrombin receptor (PAR4) is activated, and antiangiogenic proteins are released, leading to the maturation and stabilization of the vasculature (Italiano et al. 2008). In contrast, oncogenic induction of the stroma and inflammatory cells leads to continuous release of proangiogenic proteins. A lack

of antiangiogenic proteins, which leads to the maturation and stabilization of the vasculature, results in an immature and a highly unstable tumor vasculature (Klement et al. 2013). In addition, it has been reported that stimulation with ADP or a breast cancer cell line, MCF-7, results in the release of proangiogenic VEGF from platelets, whereas stimulation with thromboxane A2 results in the release of antiangiogenic endostatin. These reports suggest that angiogenesis is tightly regulated by various G protein-coupled receptor-mediated signals depending on the physiological/pathological environment. However, the concept that pro-/antiangiogenic proteins are separated in distinct subpopulations of α-granules and released upon PAR1/PAR4 stimulation has been recently challenged by evidence obtained from quantitative immunofluorescence assays and micro-ELISA arrays to quantify the release of distinct α-granule cargo molecules (Kamykowski et al. 2011; Jonnalagadda et al. 2012; Etulain et al. 2015). Further investigations are required to reveal the mechanism of regulation for pro-/antiangiogenic factor release and determine the in vivo conditions under which the selective stimulation of various platelet receptors may occur.

In addition to endothelial cell proliferation and migration, the angiogenic process requires bone marrow-derived cell (BMDC) recruitment to sites of neovascularization. Jin et al. reported that hypoxia-induced thrombopoietin induces the release of SDF-1 from platelets and that released SDF-1 recruits nonendothelial CXCR4+ VEGFR1+ hematopoietic progenitors, which enhances neovascularization (Feng et al. 2011). Feng et al. also reported that secretion of platelet α-granules, but neither dense granule secretion, nor platelet aggregation, is crucial for BMDC homing and subsequent angiogenesis using hypoxia-induced angiogenesis models (Feng et al. 2011).

Thus, platelet granule contents contribute to angiogenesis by increasing the proliferation and migration of endothelial cells and by recruiting bone marrow-derived hemangiocytes, mainly through the secretion of α-granule contents.

Contributions of Platelet Membrane Proteins to Angiogenesis

Kisucka et al. showed that mice lacking extracellular GPIbα, but not the mouse model of platelet storage pool deficiency ($Lyst^{bg}$), experienced a significant reduction in corneal neovascularization and developed hemorrhages at sites of angiogenesis using the cornea micropocket assay and the Matrigel model (Kisucka et al. 2006). These findings suggest that adhesive interactions between platelets and angiogenic vessels, but not dense-granule contents, stabilize developing blood vessels, thereby supporting neoangiogenesis. GPIbα is the receptor for von Willebrand factor as well as other adhesive and procoagulant proteins. Platelet–vessel wall interactions may involve platelet adhesion to the extracellular matrix and/or intact activated endothelium. However, both P-selectin-/- mice and VWF-/- mice exhibit normal angiogenesis without hemorrhages, suggesting that GPIbα ligands other than VWF and P-selectin are responsible for the stabilization of developing vessels. Rhee et al. reported that a highly specific antagonist of integrin $\alpha_{IIb}\beta_3$, which is a fibrinogen and VWF receptor on the surface of platelets, resulted in an approximate 35–50 % reduction in retinal neovascularization, and that platelet remnants and microvesicles were found at sites of angiogenic sprouts. Thus, platelet adhesive receptors, including GPIbα and αIIbβ3, contribute to the stabilization of developing vessels, thereby facilitating neoangiogenesis.

The Role of Platelets in Maintaining the Integrity of High Endothelial Venules in Lymph Nodes

Circulating lymphocytes continuously enter lymph nodes for immune surveillance through specialized blood vessels called high endothelial venules (HEVs). This process increases markedly during immune responses, but no bleeding from HEVs occurs during this process. Herzog et al. identified the mechanism, in which HEVs permit lymphocyte transmigration while maintaining vascular integrity (Herzog et al. 2013). Podoplanin expressed on fibroblastic reticular cells, which surround HEVs, stimulates platelets by binding to its receptor, CLEC-2. Sphingosine-1-phosphate released from activated platelets promotes VE-cadherin expression on HEVs, which is essential for overall vascular integrity. Mice deficient in CLEC-2, podoplanin, or sphingosine-1-phosphate exhibit spontaneous bleeding in mucosal lymph nodes and bleeding in the draining peripheral lymph nodes after immunization. Thus, platelet activation via CLEC-2 is necessary for maintenance of HEV integrity during immune responses (Fig. 3A).

The Role of Platelets in Maintaining the Integrity of Vessels During Inflammation

Platelets have been long known to maintain the integrity of the vasculature (Danielli 1940). Danielli et al. showed that edema in perfused frog hind legs was reduced when platelets were added to the perfusate medium, suggesting that platelets contribute to the maintenance of vascular integrity. Petechial hemorrhage is sometimes observed in severe thrombocytopenia even without traumatic injury. However, the role of platelets in the maintenance of vascular integrity is particularly important during inflammation, when

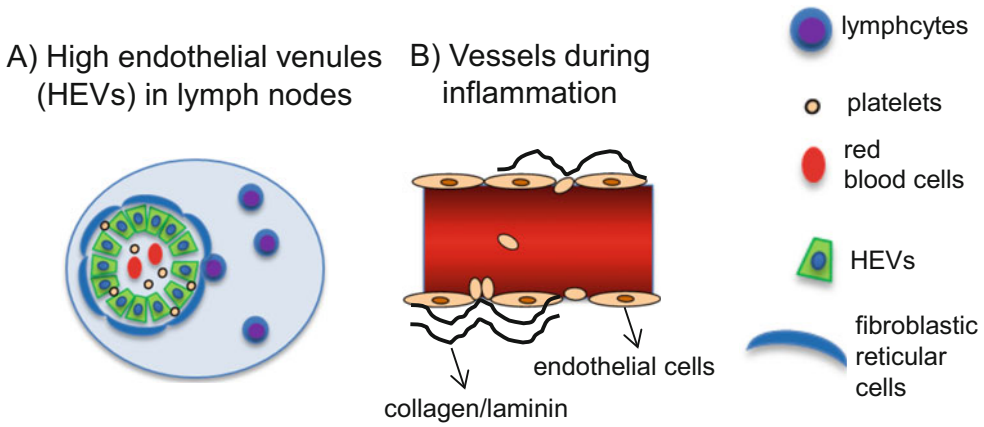

Fig. 3 Maintenance of vascular integrity by platelets. (**a**) Maintenance of the integrity of high endothelial venules in lymph nodes: podoplanin expressed on fibroblastic reticular cells, which surround HEVs, stimulates leaking platelets by binding to CLEC-2. Sphingosine-1-phosphate released from activated platelets promotes the expression of VE-cadherin on HEVs, which is essential for vascular integrity. (**b**) Maintenance of the integrity of inflammatory vessels: during inflammation, inflammatory cytokines enhance vascular permeability, and platelets leak from vessels during inflammation when vascular permeability is increased. GPVI signaling is activated at sites of inflammation by collagen and/or laminin, the two physiological ligands for GPVI found in the vessel wall. Platelet CLEC-2 may interact with podoplanin on the surface of infiltrating macrophages or an as yet unidentified ligand expressed in tissues around vessels

inflammatory cytokines enhance vascular permeability. Goerge et al. showed that acute severe thrombocytopenia in mice does not lead to hemorrhage unless the animals are challenged with an inflammatory insult (Goerge et al. 2008). They also showed that thrombocytopenia resulted in hemorrhage only at the site of inflammation and that GPIb-V-IX and integrin αIIbβ3, which are necessary receptors for platelet aggregation/agglutination, were not required for the maintenance of vascular integrity. These findings suggest that platelets do not maintain vascular integrity by forming a hemostatic plug, and exactly how platelets maintain vascular integrity during inflammation is unclear. Several studies proposed that granule contents released from activated platelets may prevent hemorrhage by strengthening endothelial barrier function or inhibiting inflammation (Boulaftali et al. 2014). A number of candidates for granule contents that regulate angiogenesis have been proposed, including ADP, serpins (protease nexin-1 and plasminogen activator inhibitor I), metalloproteinases, reactive oxygen scavengers, TREM-like transcript-1 shed from the surfaces of platelets, and sphingosine-1 phosphate (McGarrity et al. 1988a, b; Booth et al. 1988; Boulaftali et al. 2010; Villeneuve et al. 2009; Washington et al. 2009; Fong et al. 2011; Yatomi et al. 1997). However, how platelets are activated and release granule contents following vascular injury and the extent of the contribution of these factors to the maintenance of vascular integrity during inflammation are not known.

An elegant study by Boulaftali et al. proved that ITAM signaling, but not G protein-coupled receptor GPCR signaling, is critical for the prevention of inflammation-induced hemorrhage (Boulaftali et al. 2013). In their study, thrombocytopenia was induced by injection of antibodies that recognize the human interleukin-4 (IL-4) receptor into transgenic mice whose platelets express a chimeric protein consisting of the extracellular domain of the human IL-4 receptor and intracellular domain of murine GPIbα. These thrombocytopenic mice were transfused with wild-type platelets, platelets deficient in surface receptors, or antiplatelet drug-treated platelets, which are not destroyed by the circulating antihuman IL-4 receptor antibodies. Inflammation-induced hemorrhage in thrombocytopenic mice was rescued by transfusion of wild-type platelets, thrombin receptor-deficient platelets, or aspirin/clopidogrel-treated platelets, but not by transfusion of platelets deficient in CLEC-2, GPVI, or SLP-76 (a signaling molecule downstream of CLEC-2 and GPVI). These results indicate that the control of vascular integrity is a major function of immune-type receptors on platelets. On the other hand, signaling from GPCR, including thrombin receptors, thromboxane A2 receptors, and ADP receptors, is not necessary for controlling vascular integrity, whereas it is quite important for hemostasis. It has been reported that bleeding time is significantly prolonged in humans or mice with platelets deficient in signaling from GPCR (Mielke 1982; Bird et al. 2011; Fabre et al. 1999; Hamilton et al. 2004). On the other hand, GPVI/FcRγ-chain-deficient mice or CLEC-2-deficient mice do not show significant prolongation of bleeding time (Suzuki-Inoue et al. 2010; Bender et al. 2013; Hughes et al. 2010), suggesting that signaling from ITAM receptors is not required for hemostasis but is required for maintenance of vascular integrity.

As for the mechanism, it is assumed that platelets leak from vessels during inflammation when vascular permeability is increased and that GPVI signaling is likely activated at sites of inflammation by collagen and/or laminin, the two physiological ligands for GPVI found in the vessel wall

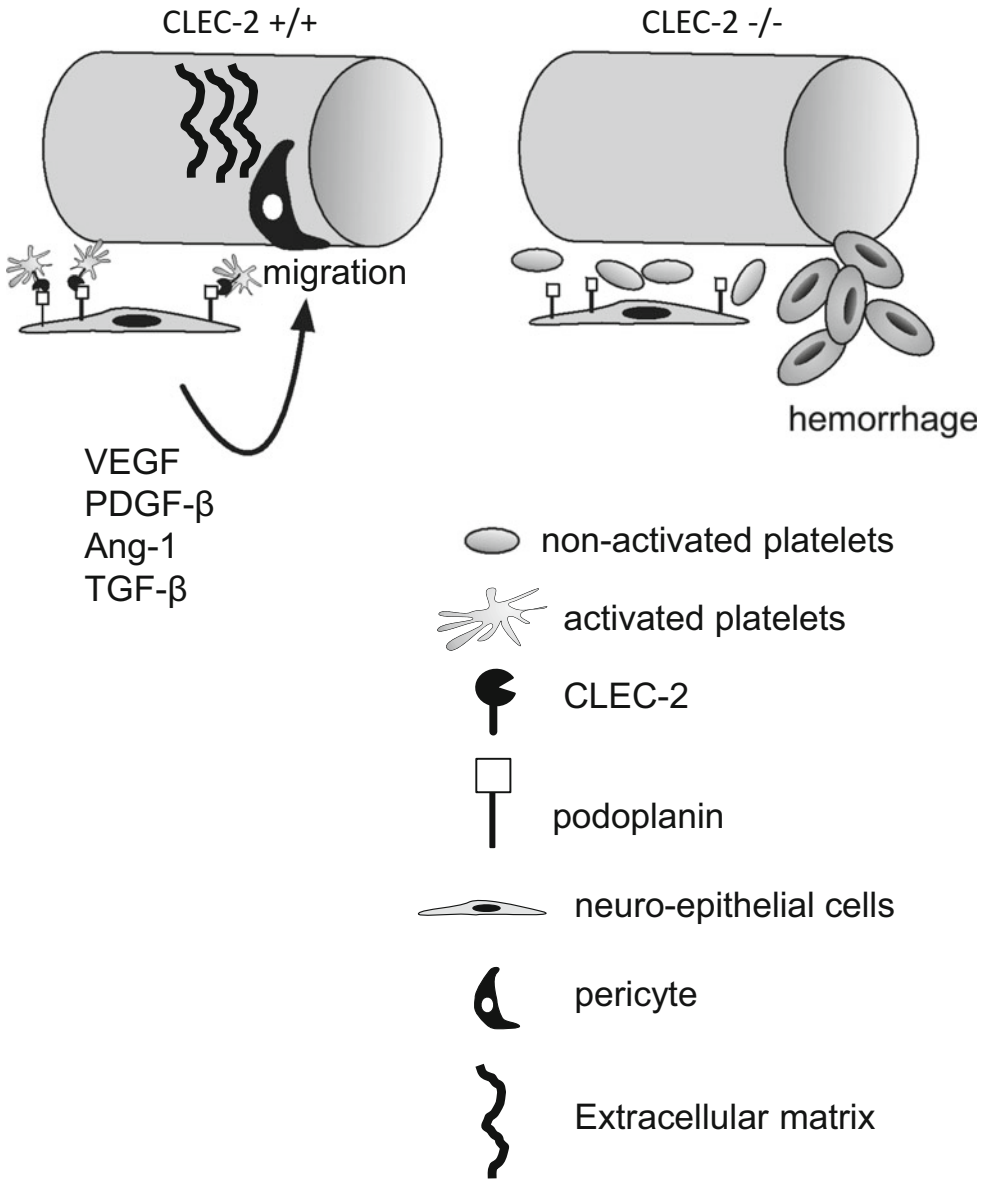

Fig. 4 Cerebrovascular patterning and maintenance of integrity by platelets during development. In CLEC-2+/+ mice, podoplanin on the neuroepithelial cells surrounding cerebral vessels interacts with CLEC-2 on the surface of leaking platelets, mediating platelet adhesion, aggregation, and secretion. Platelet granule contents recruit pericytes, and the recruited pericytes produce extracellular matrix to guide the maturation and integrity of the developing vasculature and prevent hemorrhage. In CLEC-2-/- mice, platelets are not activated by neuroepithelial cells, resulting in the absence of pericytes and extracellular matrix. As a result, cerebral vessels are tortuous and aberrantly patterned at E10.5 in CLEC-2-deficient mice, preceding the formation of large brain hemorrhages

(Ozaki et al. 2009; Inoue et al. 2006). How platelets are activated through CLEC-2 at the site of inflammation is difficult to explain, but Boulaftali et al. speculate that podoplanin on the surface of infiltrating macrophages, or an as yet unidentified ligand expressed in tissues around vessels or in the vessel wall, stimulates platelets via CLEC-2. Alternatively, podoplanin expression may be induced in the tissue around the vessels by inflammatory cytokines (Fig. 4). However, it is unclear whether this is due to the formation of an integrin αIIbβ3-independent hemostatic plug or modulation of endothelial function. Further studies are required to clarify the mechanism.

The Role of Platelets in Cerebrovascular Patterning and Integrity During Development

CLEC-2 in platelets plays a crucial role not only in neoangiogenesis and maintenance of vascular development in adults, but also in cerebrovascular patterning during development. Mice with a constitutive or platelet-specific deletion of CLEC-2 exhibit hemorrhaging in the brain at mid-gestation. Lowe et al. reported that cerebral vessels were tortuous and aberrantly patterned at E10.5 in podoplanin- and CLEC-2-deficient mice, preceding the formation of large brain

hemorrhages. They proposed that podoplanin on the neuroepithelium, which surrounds cerebral vessels, interacts with CLEC-2 on leaking platelets, mediating platelet adhesion, aggregation, and secretion. Platelet granule contents recruit pericytes, and the recruited pericytes produce extracellular matrix to guide the maturation and integrity of the developing vasculature and prevent hemorrhage (Fig. 4) (Lowe et al. 2015). Thrombocytopenia is the most common risk factor for intraventricular hemorrhage (IVH) in premature infants (Ballabh 2010). It was recently reported that platelet infusions in high-risk preterm infants enhance hemostasis and reduce the risk of IVH (Coen 2013). The observations by Lowe et al. may provide a potential explanation for IVH in premature infants and therapeutic insights (Lowe et al. 2015).

Take Home Messages

- Platelets facilitate blood/lymphatic vessel separation during development through the association between CLEC-2 on platelets and podoplanin on lymphatic endothelial cells.
- Angiogenesis activators and inhibitors contained in platelet alpha granules are released upon platelet activation and delivered to the site of neoangiogenesis.
- Platelets maintain the integrity of high endothelial venules (HEVs) in lymph nodes through the association between CLEC-2 on platelets leaking from HEVs and podoplanin on fibroblastic reticular cells.
- Platelets maintain the vessel integrity during inflammation, for which the ITAM-containing receptors, GPVI and CLEC-2 are
- Platelets facilitate proper cerebrovascular patterning during development through the association between CLEC-2 on platelets and podoplanin on the neuroepithelium.

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Platelets and Coagulation

Johan W.M. Heemskerk, Judith M.E.M. Cosemans, and Paola E.J. van der Meijden

Abstract

Platelet activation and blood coagulation are reciprocal processes, both contributing to hemostasis and thrombosis in an interactive way. Exposed collagen, von Willebrand factor (VWF), and other components of the subendothelial matrix initiate platelet adhesion, whereas exposed tissue factor simultaneously triggers the coagulation cascade. During the various phases of the coagulation process, activated platelets support and steer coagulation by at least four mechanisms, namely, by (1) exposing the procoagulant phospholipid phosphatidylserine (PS) at their outer surface; (2) releasing coagulation factors like factors V and XIII, as well as anticoagulant factors like tissue factor pathway inhibitor; (3) providing a scaffold for the formation of fibrin fibers; and (4) stimulating retraction of the fibrin clot. In this chapter, we describe these roles of platelets in detail, also focusing on the receptors and signaling pathways involved and on the distinct (anti)coagulant pathways.

Platelets in the Stages of Coagulation

Traditionally, hemostasis and, by implication, also arterial thrombosis are considered as a two-stage processes of platelet plug formation (primary hemostasis) or platelet thrombus formation followed by fibrin clot generation (secondary hemostasis). However, in the last decades, it has become clear that platelet activation and blood coagulation are highly reciprocal processes, each contributing to hemostasis and thrombosis in an interactive way. The initiation of platelet adhesion is considered to start by exposed vascular collagen, von Willebrand factor (VWF), and other components of the subendothelial matrix, while exposed tissue factor (TF) simultaneously triggers the coagulation cascade.

This insight of continued interactions between platelets and coagulation activation has led to new concepts of cell-controlled coagulation with separate roles for TF-bearing cells and platelets (Mackman 2008; Jackson 2011; Versteeg et al. 2013). Herein, the coagulation process takes place in various phases (Mackman 2008): (1) an initiation phase of TF expression and generation of small amounts of activated factor (F)VII, FIX, and FX; (2) an amplification phase in which platelets and VWF (bearing FVIII) take care of increased procoagulant activity, resulting in the activation of FX (via activated FVIII and FIX) and the activation of prothrombin (via activated FV and FX); (3) feeding back of the formed thrombin, e.g., to activate FXI; (4) a propagation phase where massive quantities of thrombin and fibrin are formed; (5) and the extension phase where continued thrombin activity ensures platelet activation, clot remodeling and fibrinolysis, and wound healing.

During the various phases of the coagulation process, activated platelets can support and steer the coagulation process by at least four mechanisms (Heemskerk et al. 2013; De Witt et al. 2014a), namely, by (1) exposing the procoagulant phospholipid phosphatidylserine (PS) at their outer surface; (2) releasing coagulation factors like FV and FXIII, as well as anticoagulant factors like tissue factor pathway inhibitor (TPFI); (3) providing a scaffold for the formation of fibrin fibers; and (4) stimulating retraction of

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the fibrin clot. Below we describe these coagulant roles of platelets in more detail.

Mechanisms of Platelet PS Exposure

Negatively charged phospholipid membranes, in particular with surface-exposed phosphatidylserine, appear upon platelet activation (via elevated cytosolic Ca^{2+}), during apoptosis (Ca^{2+} independently via caspases), or upon cell injury/necrosis (causing lysis) (Jackson and Schoenwaelder 2010). As reviewed in detail elsewhere (Heemskerk et al. 2013), combinations of strong platelet agonists, such as collagen/thrombin or Ca^{2+} ionophores, cause a prolonged elevation in cytosolic Ca^{2+} level, which is a direct prerequisite for PS exposure. Predominant pathways responsible for this are signaling via the glycoprotein VI-Fc receptor γ-chain (GPVI-FcRγ) complex, phospholipase Cγ, and phosphatidylinositol 3-kinase (PI3K) isoforms (Heemskerk et al. 2013; Munnix et al. 2009). For the sustained Ca^{2+} response, mobilization of Ca^{2+} from various sources is required, i.e. Ca^{2+} release from the endoplasmic reticulum, entry of extracellular Ca^{2+} via the Orai1 channel and the STIM1 sensor, and Ca^{2+} liberation from the mitochondria. The Ca^{2+}-dependent scrambling of membrane phospholipids, required for PS exposure, is mediated by the Ca^{2+}-dependent ion channel protein anoctamin-6 (see below). Platelets from a patient with Scott syndrome, lacking this protein, are greatly impaired in agonist-induced PS exposure, but have only a minor defect in apoptosis-induced PS exposure (Van Kruchten et al. 2013).

The PS-exposing platelet membranes of activated platelets form high-affinity binding sites for vitamin K-dependent coagulation and anticoagulation factors, i.e., prothrombin, FVII, FIX, FX, protein C, S, and Z (Zwaal and Schroit 1997; Monroe et al. 2002). Gamma-carboxyglutamate (Gla) domains, which are present in all vitamin K-dependent proteins, mediate their binding to PS membranes in a Ca^{2+}-dependent manner. As a consequence, the PS-exposing platelets—as well as PS-exposing platelet-derived microparticles—serve as assembly sites for components of the tenase complex (FVIIIa and FIXa, activating FX) and the prothrombinase complex (FVa and FXa, activating prothrombin). Studies with isolated coagulation factors have shown that PS-containing membranes enhance the activities of both the tenase and prothrombinase complexes by up to 1000-fold in comparison to absence of phospholipids (Bevers et al. 1991; Heemskerk et al. 2002). Clearly, in hemostasis also PS-exposing membranes from other (damaged) cell types can supply such a procoagulant surface and thus support thrombin generation. Recent work supports this for a damaged/activated endothelium (Ivanciu et al. 2014).

Mechanisms of Platelet Retraction and Clot Contraction

Classically, clot retraction is regarded as a platelet response induced by $\alpha_{IIb}\beta_3$ outside-in signaling, thus implying dependency of (thrombin-induced) $\alpha_{IIb}\beta_3$ activation and binding of fibrin(ogen) to platelets (Shattil et al. 2010). The outside-in signaling process leads to actin and myosin cytoskeleton rearrangements, which are needed for the generation of contraction forces that directly transmit to the fibrin clot, causing it to condense (Martin et al. 2010; Schoenwaelder et al. 2010). Mechanistic studies also point to roles of Rho and Src family kinases in the contraction of platelets present in an aggregate or thrombus, which precede the actin-dependent process and can even occur in the absence of thrombin or a fibrin clot (Ono et al. 2008; Auger and Watson 2008). As reviewed elsewhere, multiple studies with genetically modified mice indicate that defective integrin activation almost invariably is accompanied by an impairment of clot retraction (De Witt et al. 2014a).

The cytoskeletal rearrangements required for platelet contraction and clot retraction appear to be hampered in PS-exposing platelets, most likely due to calpain-mediated degradation of the actin cytoskeleton (Mattheij et al. 2013). This can explain why PS-exposing platelets do not contribute to clot retraction.

Mechanisms of Platelet-Dependent Thrombin and Fibrin Generation

As detailed below, PS exposure is both required and sufficient for massive generation of active FXa and thrombin at the platelet surface, e.g., as exemplified in thrombin generation experiments (Vanschoonbeek et al. 2004). Such PS-exposing platelets also produce fibrin fibers at their surface (Cosemans et al. 2011) where, strikingly, the fibrin acts as a main sink of (proteolytically active) thrombin, likely contributing to the further growth of these fibers (Berny et al. 2010). This can provide a mechanism for the earlier recognized positive feedback loops of thrombin, fibrin, and platelet activation (Béguin and Kumar 1997). In line with this observation, patients with complete fibrinogen deficiency can develop thrombosis, which can originate in both the arterial or venous territories (Girolami et al. 2012). A reasonable explanation for this thrombotic tendency is that the formed thrombin in these patients is more active, since it cannot be sequestered by binding to fibrin (De Bosch et al. 2002).

Especially in vivo studies have pointed to a different way of regulation of platelet-dependent coagulation at high, arterial and low, venous flow conditions. At high wall shear rates, where platelet deposition is enforced by interaction

of glycoprotein(GP)Ib-V-IX with VWF, the formation of a platelet-rich (white) thrombus predominates, whereas in regions of low shear flow, more fibrin fibers are formed (Jackson 2011). Also the roles of platelet receptors for collagen (GPVI) and ADP ($P2Y_{12}$) increase at higher shear rates (Nergiz-Unal et al. 2010). The limited evidence so far indicates that the role of thrombin receptors (PARs) in platelet activation decreases at high shear conditions (Lee et al. 2012). Recent in vitro findings indicate that the diminished formation of thrombin and fibrin at higher shear rate can be explained by thrombin dilution during the blood flow, which implies that also at high shear rates thrombin generation is a limiting factor in thrombus formation (Okorie et al. 2008; Berny et al. 2010). Since signaling via both GPVI and PARs can lead to PS exposure, it is understandable that this platelet response is relatively independent of the shear flow conditions (Munnix et al. 2005).

Neither thrombin nor fibrin appears to be uniformly distributed in an arterial thrombus. In vivo studies in the arterial circulation point to a thrombus core with densely packed platelets, in which thrombin is retained, that is surrounded by layers of platelets with reduced activation (Welsh et al. 2012; Stalker et al. 2014). The PS-exposing platelets appear as separate patches in such thrombi (Munnix et al. 2007). Interestingly, it appears that fibrin initially accumulates at the thrombus base and, at later time points, depending on the "coagulant force", gradually builds up within and outside the thrombus (Furie and Furie 2005; Swieringa et al. 2016). In mouse laser injury models, where typically vascular TF becomes exposed, also the injured vascular membranes contribute to the prothrombinase activity (Ivanciu et al. 2014).

Roles of ITAM and hem-ITAM Receptors

Platelet receptors signaling via protein tyrosine kinases are intricately involved in coagulation stimulation. In particular, this holds for receptors acting through the so-called ITAM motif (two adjacent YxxL sequences) or hem-ITAM motif (one YxxL sequence), both of which rely on tyrosine phosphorylation in order to transmit signals. Such receptors are the immunoglobulin receptor GPVI, the FcRIIA receptor, and the C-type lectin receptor CLEC-2.

For long, *GPVI* has been known as the central signaling receptor for collagen (Nieswandt and Watson 2003). However, evidence is accumulating that it can also recognize laminin (Schaff et al. 2013) and fibrin fibers (Mammadova-Bach et al. 2015; Alshehri et al. 2015), thus placing GPVI at a central position in the interactions between platelet and coagulation activities. Deficiency in GPVI leads to an impairment not only of collagen-induced platelet adhesion and aggregation but also of PS exposure (Heemskerk et al. 1997b). On the other hand, GPVI deficiency is accompanied by only a mild bleeding phenotype (Moroi et al. 1989), which may point to functional redundancy of GPVI with other platelet receptors (Shida et al. 2014).

GPVI in association with the FcR γ-chain signals via a signalosome containing several protein tyrosine kinases (Src family and Syk kinases). This results in phospholipase Cγ activation, followed by a prolonged elevation in cytosolic Ca^{2+} (Watson et al. 2005). Analysis of mice lacking GPVI or other proteins of the GPVI signalosome indicates that the assembly of multiple tyrosine kinase-linked proteins is essential for high shear thrombus formation and Ca^{2+}-dependent PS exposure on a collagen surface (Munnix et al. 2005; Nieswandt et al. 2001; De Witt et al. 2014a). Since GPVI is only a low-affinity receptor for collagen, platelet-collagen adhesion needs to be enhanced by co-receptors like integrin $\alpha_2\beta_1$ and GPIb-V-IX (Siljander et al. 2004).

Negative feedback between coagulation and platelet activation takes place via shedding of GPVI on activated platelets by the extracellular proteases ADAM-10 and ADAM-17, which abrogates GPVI-induced responses (Bender et al. 2010). Interestingly, at least part of this proteolytic cleavage is regulated in a not well-understood way by FXa, but not by thrombin (Al-Tamimi et al. 2011).

Clustering of the platelet hem-ITAM receptor, *CLEC-2*, similarly occurs via the Syk tyrosine kinase route (Watson et al. 2010). Platelet stimulation via CLEC-2 relies in part on ADP- and thromboxane A_2-dependent signaling pathways (Pollitt et al. 2010). Known ligands for CLEC-2 are podoplanin, which however is not present in the vasculature, and the snake venom protein rhodocytin. In spite of unclear knowledge of the ligand in vivo, mouse studies have shown that CLEC-2 is an essential platelet-activating receptor in hemostasis and thrombosis (May et al. 2009). In agreement with this notion, platelet interaction with immobilized CLEC-2 ligands leads to full thrombus formation and substantial PS exposure, in a way resembling platelet activation via GPVI (De Witt et al. 2014b).

The immunoglobulin receptor *FcγRIIA*, activated by clustering with IgG immunoglobulins, also evokes Syk-dependent signaling pathways in human platelets, again enforced by autocrine stimulation (Lhermusier et al. 2011). This can lead to thrombocytopenia; to which extent platelet procoagulant activity is increased is still unclear.

Roles of Other Platelet Adhesive Receptors

Interaction of the *GPIb-V-IX* receptor with VWF is one of the first steps in shear-dependent platelet adhesion and, as such, a requirement for the procoagulant role of platelets under high shear conditions. GPIb-V-IX with ~25,000 copies present on platelets induces by itself only weak intracellular signals,

activating Src-related protein kinases, phosphoinositide 3-kinases (PI3K), and small GTPases. These events cause only small Ca^{2+} fluxes, limited integrin $\alpha_{IIb}\beta_3$ activation, and platelet spreading (Canobbio et al. 2004). However, under coagulant conditions, there is evidence that the GPIb-V-IX complex, by interaction with VWF, enhances platelet PS exposure and thereby the formation of thrombin (Béguin et al. 1999; Weiss 2009). Furthermore, GPIb-V-IX appears to have a broader role than only serving as VWF receptor. It binds thrombin with high affinity, which supports activation of the thrombin receptors PAR1 and PAR4 (De Candia et al. 2001). In addition, GPIbα has been proposed to interact with several coagulation factors, such as FVII, FXI, FXII, and high molecular weight kininogen, although the functional consequences of these interactions are unclear (Heemskerk et al. 2013). One can conclude that it is this combination of properties that makes GPIb-V-IX such a crucial receptor in thrombus formation (Bergmeier et al. 2008).

Integrin $\alpha_{IIb}\beta_3$ (*GPIIb/IIIa*) is the most abundant platelet membrane glycoprotein with ~80,000 copies per platelet, and additional copies present in the open canicular system and α-granules. Integrin outside-in signaling, via Src and Syk tyrosine kinases, regulates the spreading of platelets over a fibrinogen surface and mediates the retraction of a platelet-fibrin clot (De Witt et al. 2014a). As a persistent process, signaling via activated integrin $\alpha_{IIb}\beta_3$ (outside-in signaling) is implicated in the coagulation process in various ways. Outside-in signaling, via prolongation of intracellular Ca^{2+} rises, contributes to agonist-induced PS exposure (Van der Meijden et al. 2012). This pathway is however restricted by a negative feedback mechanism, because PS-exposing platelets with high Ca^{2+} levels gradually close their integrins which abrogates the outside-in signaling (Cosemans et al. 2008). The underlying mechanism of this signaling inactivation is calpain-mediated cleavage of multiple integrin-associated proteins including Src and talin (Mattheij et al. 2013).

As an adhesive collagen receptor, *integrin $\alpha_2\beta_1$* sustains and enforces human platelet adhesion to collagen/VWF under high shear conditions via GPIb-V-IX and GPVI (Siljander et al. 2004; Ruggeri 2002). Since platelets in contact with immobilized $\alpha_2\beta_1$-binding peptides are hardly activated, the integrin may primarily support platelet activation via the other collagen receptor, GPVI (Munnix et al. 2008). In a similar way, the laminin receptor integrin $\alpha_6\beta_1$ may also support platelet activation and PS exposure under flow conditions (De Witt et al. 2014b).

The glycoprotein *CD36* (*GPVI*, *GPIIIb*) is abundantly expressed on platelets (10,000–25,000 copies). On platelets it may have different roles, but especially acts as a receptor for thrombospondin-1 and oxidized low-density lipoproteins (Nergiz-Unal et al. 2011; Podrez et al. 2007). CD36 has a limited contribution to platelet procoagulant activity, especially under low shear conditions of thrombus formation (De Witt et al. 2014b). The underlying signaling pathway likely is by enforcing protein tyrosine kinase activation and subsequent Ca^{2+} rises (Nergiz-Unal et al. 2011).

Roles of Receptors for Soluble Agonists (Thrombin, ADP)

As a Gla-domain coagulation factor, prothrombin binds to the membrane of PS-exposing platelets (Versteeg et al. 2013). However, once cleaved by the prothrombinase complex (FXa, FVa), the formed α-thrombin interacts with PAR receptors with high affinity and massively translocates to fibrin fibers (Berny et al. 2010). The conventional concept is that fibrin-bound thrombin is protected from inactivation by antithrombin and the heparin-antithrombin complex (Weitz et al. 1990), but as a result this sequestering will locally confine the thrombin to the direct environment of the clot. Thrombin is a key protease in the coagulation cascade and is hence required for fibrin formation. It is a moderately strong platelet agonist, which enforces PS exposure in combination with other agonists such as GPVI ligands (Mattheij et al. 2016).

The Gq-protein-coupled receptors for thrombin on platelets, i.e., the protease-activated receptors *PAR1* and *PAR4* in human platelets and *PAR3* and *PAR4* in mouse platelets, accomplish full integrin activation and secretion (Offermanns 2006). Thrombin-induced activation of human platelets is dependent on PAR1, since this receptor displays a higher affinity to thrombin than PAR4. The latter is considered to enhance the action of PAR1, e.g., at higher thrombin concentrations (Coughlin 2005; Andersen et al. 1999). Signaling via the Gq protein and phospholipase Cβ, thrombin elicits oscillatory rises in cytosolic Ca^{2+} (Heemskerk et al. 1997a), thus explaining why thrombin possesses limited PS exposure and procoagulant activity on its own. However, thrombin by itself via the PAR receptors greatly enforces the PS exposure induced by collagen receptors (Heemskerk et al. 2002). The likely mechanism for this is potentiation of the GPVI-induced Ca^{2+} signal (Keuren et al. 2005). Strikingly, the limited PS exposure induced by even high thrombin concentrations is less dependent on Ca^{2+} entry via STIM1 and Orai1 (Gilio et al. 2010), but rather relies on receptor-operated Ca^{2+} entry via TRPC channels (Harper et al. 2013). Next to PAR1, also PAR4 has a potentiating role in thrombin-induced procoagulant activity (Dorsam et al. 2004). In mouse platelets, both PAR3 and PAR4 appear to be required for the thrombin effect on PS exposure (Coughlin 2000).

There is limited evidence that the PARs, especially PAR1, can be cleaved by other proteases than thrombin, such as ADAM-17 and matrix metalloproteinase-1 (Andrews et al. 2007; Trivedi et al. 2009), activated

protein C, and neutrophil-derived cathepsin G and the fibrinolysis protease plasmin cleaving PAR4 (Quinton et al. 2004; Sambrano et al. 2000). The relevance of these alternative pathways of thrombin receptor cleavage for platelet procoagulant activity remains to be determined.

The platelet dense granules contain high concentrations of the adenosine nucleotides *ADP* and *ATP*. Once secreted, ADP enhances platelet activation via interaction with the purinergic receptors *P2Y$_1$* and *P2Y$_{12}$*. Similarly to the PARs, the $P2Y_1$ receptor couples to Gq leading to oscillatory Ca^{2+} mobilization through phospholipase Cβ stimulation (Heemskerk et al. 2001). The $P2Y_{12}$ receptor signals via a Gi-coupled pathway that lowers cyclic AMP and stimulates the PI3K route (Versteeg et al. 2013). In addition to a role in (sustaining) platelet aggregation, ADP appeared to potentiate tissue factor-triggered thrombin generation, which effect was assigned to stimulation of the $P2Y_{12}$ and not the $P2Y_1$ receptors (Dorsam et al. 2004; Van der Meijden et al. 2005). Signaling via $P2Y_{12}$ receptors prolongs the platelet Ca^{2+} rises as a consequence of (1) enhanced Ca^{2+} mobilization by the 1,4,5-triphosphate receptors due to inhibition of protein kinase A and (2) prolonged activation of PI3K (Van der Meijden et al. 2008). There is also evidence that $P2Y_{12}$ regulates the $P2Y_1$-induced Ca^{2+} responses via inhibition of adenylate cyclase and activation of PI3K (Hardy et al. 2004). In line with these signaling schemes, inhibition of the $P2Y_{12}$ receptors in patients taking clopidogrel significantly suppressed PS exposure of the platelets in coagulating plasma (Van der Meijden et al. 2005). Similarly, the active metabolite of prasugrel (another $P2Y_{12}$ receptor antagonist) also impairs collagen-induced and $P2Y_{12}$-dependent Ca^{2+} rises, PS exposure, and thrombin generation in coagulant whole blood (Frelinger et al. 2008; Judge et al. 2008).

Thromboxane A$_2$ is a well-known autocrine mediator for platelet activation and aggregation. It interacts with the *TP* receptors which, like the PARs, are coupled to Gq but to a lower extent (Offermanns 2006). Stimulation of TP receptors leads to limited Ca^{2+} mobilization and is therefore hardly effective in mediating platelet PS exposure (Swieringa et al. 2014). In agreement with this, it appeared that blockage of thromboxane A_2 formation by aspirin treatment only slightly affects thrombin generation in platelet-rich plasma (Vanschoonbeek et al. 2004). In platelets adhering to collagen, aspirin decreased PS exposure if used in combination with ADP receptor blockers (Lecut et al. 2004).

Roles of Contact-Dependent Signaling Receptors

Platelets also communicate via homotypic receptors, which have collectively been termed as contact-dependent signaling receptors. From studies with genetically modified mice, it has been deduced that such homotypic interactions contribute in a balanced (positive and negative) way to platelet clot retraction (De Witt et al. 2014a). Thus, the *connexins* 37 and 40, implicated in gap junction formation, have a stimulatory effect on platelet-dependent clot retraction (Vaiyapuri et al. 2012, 2013). Connexins also contribute to other activation processes, such as platelet aggregation, integrin activation, Ca^{2+} signaling, and α-granule secretion (Vaiyapuri et al. 2012).

Similarly, the *tetraspanin members* 32 (TSSC6) and CD151 enhance clot retraction (Goschnick et al. 2006; Lau et al. 2004). Interestingly, in CD151 knockout mice, blockage of the $P2Y_{12}$ receptors was found to lead to an extra defect in clot retraction, suggesting that CD151 and $P2Y_{12}$ have a complementary role in modulating integrin $\alpha_{IIb}\beta_3$ outside-in signaling (Makkawi et al. 2015). More research will be needed to elucidate the action mechanisms of tetraspanins in platelet-based coagulation.

Junctional adhesion molecule (*JAM*)*-A* acts as negative regulator of integrin $\alpha_{IIb}\beta_3$ outside-in activation and clot retraction (Naik et al. 2012). The proposed mechanism is suppression by JAM-A of integrin outside-in signaling through downstream protein kinases such as Src, Syk and focal adhesion kinase (Obergfell et al. 2002), and via phosphatases like PTPN1 (Karshovska et al. 2015).

In some instances, contact signaling receptors seem to act differently on integrin outside-in signaling and on clot retraction. In platelets lacking *endothelial cell-specific adhesion molecule* (*ESAM*), the spreading on immobilized fibrinogen (reflecting outside-in signaling) was found to be unaffected, while fibrin clot retraction was delayed (Stalker et al. 2009). Another intriguing membrane protein in terms of platelet function is *PECAM-1*. Platelets from mice lacking PECAM-1 have been reported as no more than slightly impaired in agonist-induced responses, with unchanged clot retraction (Dhanjal et al. 2007). This contrasts to a detected inhibitory role of human PECAM-1 in platelet integrin activation (Jones et al. 2014). The reason for this discrepancy is unclear.

Roles of Inhibitory Platelet Receptors

Endothelium-derived prostaglandin I_2 (prostacyclin) binds to platelets via the Gs-coupled *IP* receptors. Activation of IP receptors leads to raised intracellular cyclic AMP levels, strongly antagonizing most platelet activation processes, including PS exposure (Swieringa et al. 2014; Heemskerk et al. 1997b). Prostaglandin E_1, also raising cyclic AMP, has equal anticoagulant effects (Kuijpers et al. 2005). Similarly, the platelet inhibitory pathway induced by nitric oxide and guanylate cyclase suppresses agonist-induced PS exposure (Rukoyatkina et al. 2011). In general, the tight control of

collagen-/thrombin-induced PS exposure by multiple cyclic AMP-/GMP-inhibiting and Ca^{2+}-stimulating signaling pathways underlines the physiological importance of this platelet response (Heemskerk et al. 2013), rather than suggesting it is a nonspecific injury- or necrosis-related event (Jackson and Schoenwaelder 2010).

Prostaglandin E_2 which is produced by endothelial cells, macrophages, and platelets exerts opposing effects in comparison to prostaglandin E_1. At low concentrations, prostaglandin E_2 binds to the platelet *EP3* receptors and via Gi-dependent signaling enhances platelet responses induced by other platelet agonists (Petrucci et al. 2011; Friedman et al. 2015). At higher concentrations, it binds to *EP4* receptors, transmitting cyclic AMP-dependent and AMP-independent effects (Friedman et al. 2015).

Syndromes with Altered Platelet-Dependent Coagulation

Impaired hemostasis in patients with several well-known platelet function defects (Nurden and Nurden 2014), such as Glanzmann's thrombasthenia, is frequently accompanied by dysfunctional platelet procoagulant activity. In addition, two platelet disorders have been described that directly concern abnormalities in platelet-dependent coagulation. The Scott syndrome, a rare bleeding disorder, is characterized by mutations in the gene *ANO6* (or TMEM16F, OMIM:608663), encoding for the transmembrane ion channel protein anoctamin-6 (Suzuki et al. 2010). Platelets from Scott patients as well as from mice lacking anoctamin-6 are greatly impaired in collagen-/thrombin-induced PS exposure, swelling to rounded structures (balloons), and inactivation of integrin $\alpha_{IIb}\beta_3$ (Agbani et al. 2015; Mattheij et al. 2016b). Interestingly, the anoctamin-6 protein is also known as a Ca^{2+}-dependent ion channel conducting Cl^- and cations; it now appears that this channel also carries the negatively charged phospholipid PS (Kmit et al. 2013). Given that fact that collagen-induced PS exposure (in contrast to Ca^{2+} ionophore-induced PS exposure) is not entirely canceled in Scott platelets or mouse platelets with a complete deficiency in anoctamin-6 (Mattheij et al. 2016b), it is likely that another phospholipid scramblase activity exists.

On the contrary, an increased Ca^{2+}-dependent PS exposure has been observed in platelets from other patients with a bleeding tendency. This so-called Stormorken syndrome (or York syndrome, OMIM:185070) has been associated with a dominant mutation in the STIM1 gene, resulting in uncontrolled Ca^{2+} entry via the Orai1/STIM1 pathway (Misceo et al. 2014). In line with the increased PS exposure, the platelets from such patients are reduced in integrin $\alpha_{IIb}\beta_3$-dependent signaling events, including clot contraction.

Extrinsic Coagulation Pathway (TF, FVII)

The key trigger of the extrinsic coagulation pathway, tissue factor (TF), is a membrane protein highly expressed on cells that are not in normal contact with the bloodstream but, at a very limited amount, on blood cells. The procoagulant role of TF is highly dependent on the presence of neighboring procoagulant phospholipid membranes (Versteeg et al. 2013).

Evidence for the presence of residual TF on platelets came from the finding that TF-containing membrane vesicles can associate with activated platelets (Giesen et al. 1999). Later findings indicated that platelet activation increases the expression of (platelet-derived) TF in a form, capable to trigger thrombin generation (Müller et al. 2003). Increased TF levels on platelets coincided with the expression of P-selectin. Especially under pathological conditions, mRNA levels of TF seem to increase (Müller et al. 2003), and this mRNA is translated to active protein upon platelet stimulation (Schwertz et al. 2006; Panes et al. 2007). Since also human megakaryocytes contain TF mRNA, it is considered unlikely that the platelet mRNA is derived from other cells via a transfer mechanism (Müller et al. 2003; Schwertz et al. 2006). Recent work using in vitro cultured megakaryoblasts shows that functionally active TF is present in a subset of the shed proplatelets (Brambilla et al. 2015). The relative role of the platelet-derived TF in triggering coagulation is still unclear, especially because platelets contain and release high amounts of tissue factor pathway inhibitor (TFPI) (Maroney et al. 2011), which will effectively block any nearby surface-expressed TF.

Platelet granules contain protein disulfide isomerases, which have been implicated in the conversion (decryption) of TF into its active conformation (Versteeg et al. 2013). Secreted protein disulfide isomerases have different roles on platelets, which may or may not be linked to the decryption of TF: activation of integrin $\alpha_{IIb}\beta_3$ (Lahav et al. 2003) and platelet adhesion and fibrin formation under flow conditions (Cho et al. 2008; Jasuja et al. 2012). In agreement with this, it was observed that impaired protein disulfide isomerase secretion in Hermansky-Pudlak syndrome suppresses thrombus formation and contributes to a bleeding phenotype (Sharda et al. 2015).

The primary role of TF in coagulation is to bind FVII (a) for achieving full activation into FVIIa (Versteeg et al. 2013). Interestingly, also other binding sites of FVII(a) have been reported for platelets. These include PS-exposing membranes, which bind via its Gla-domain, and GPIbα which can capture recombinant FVIIa in platelets stimulated with collagen/thrombin and then support TF-independent thrombin generation (Weeterings et al. 2008). Furthermore, an active variant of FVIIa has been described, which displays enhanced platelet binding characteristics via the

Gla-domain and as yet undisclosed proteins (Hoffman et al. 2011). Due to its binding to platelets, recombinant FVIIa may be a suitable prohemostatic drug in patients with Glanzmann's thrombasthenia or in patients using anti-$\alpha_{IIb}\beta_3$ medication (Lisman et al. 2003).

Intrinsic Coagulation Pathway (FXI, FXII, Polyphosphates)

The intrinsic coagulation factors, *FXII* and *FXI*, play a consolidating role in platelet-dependent thrombus formation and particularly in thrombus stabilization. This has been established for in vivo mouse experiments after damage of healthy vessels (Kleinschnitz et al. 2006; Cheng et al. 2010; Revenko et al. 2011) or damage of atherosclerotic vessels (Kuijpers et al. 2014; Van Montfoort et al. 2014). Such in vivo studies collectively indicate that FXII promotes thrombus formation largely or exclusively via activation of FXI. An emerging concept is that FXII locally triggers the formation of thrombin and fibrin to stabilize specific regions in a platelet thrombus distant from the vascular-exposed TF (Kuijpers et al. 2014). Recent studies support this idea, as anti-FXII(a) antibodies were found to suppress fibrin formation in an ex vivo blood perfusion system (Larsson et al. 2014).

Although there is no strong evidence for the presence of specific binding FXII sites on platelets, a platelet-dependent role in the activation of FXII has been suspected for long. A recent postulation is that platelet-derived *polyphosphates* catalyze the FXII (auto)activation (Müller et al. 2009). However, some authors argue that, for FXII activation, polyphosphates are required with a longer-chain length than that present in platelets (i.e., 60–100 phosphates/molecule) (Smith et al. 2010; Choi et al. 2011). The shorter-chain polyphosphates seem rather to function in accelerating FV and FXI activation by thrombin and in consolidating the fibrin clot. During thrombus formation, also collagen fibers (Van der Meijden et al. 2009) and neutrophil extracellular traps (Von Brühl et al. 2012) are reported triggers of FXII activation and thus for the intrinsic coagulation pathway.

The binding of FXI to platelets has been a matter of confusion. An earlier report suggests that FXI binds via GPIbα (Baglia et al. 2004), but functional studies did finally not lead to publications. Other studies with human and mouse platelets proposed the platelet apolipoprotein E receptor 2 (ApoER2, LRP8) as a binding receptor for FXI (White-Adams et al. 2009). Detailed microscopic analysis confirmed that FXIIa does not bind to PS-exposing platelets in a thrombus, but to collagen and patches of fibrin (Kuijpers et al. 2014). This fits with the idea that fibrin acts as a scaffold for several coagulation factors (Berny et al. 2010).

Common Coagulation Pathway to Thrombin Generation

In spite of a long search, specific protein-type receptors on platelets for the vitamin K-dependent factors *FIX* and *FX* have not been identified (Heemskerk et al. 2002; Monroe et al. 2002). For instance, platelets, unlike other cell types, do not express the PAR2 receptor for FXa (Daubie et al. 2006). Investigations by fluorescence microscopy have shown that the binding of FIXa and FXa (similarly to prothrombin) is confined to PS-exposing platelets in a thrombus (Berny et al. 2010; Swieringa et al. 2015). One study proposes protein disulfide isomerases contribute to the binding of coagulation factors to thrombin-stimulated platelets and thereby stimulate the platelet-dependent thrombin generation (Jurk et al. 2011).

In platelets, *FV* is stored in the α-granules, where it comprises about 20 % of the total blood pool of FV. Megakaryocytes take up plasma FV through endocytosis (Bouchard et al. 2005). Given the high FV storage in platelets, it is likely that the secreted protein significantly contributes to the prothrombinase activity at the platelet surface (Weiss et al. 2001). It is reported by one group that platelet-derived FVa is more resistant to protease *in*activation than the FVa in plasma (Gould et al. 2004), but this finding needs confirmation. Activated FV binds to PS-exposing membranes via the C2 domain, where it assembles with FXa to form local prothrombinase complexes (Majumder et al. 2008; Berny et al. 2010). Using flow cytometry, a subpopulation of thrombin-activated platelets can be identified that is positive for both FVa and FXa binding. However, also another subpopulation was detected with intermediate FVa binding and no FXa binding, thus pointing to a second type of interaction (Fager et al. 2010). This agrees with the observation that FVa can bind in a transglutaminase-dependent way to the membrane of coated platelets (Alberio et al. 2000) and may point to a gradual accumulation of FVa at the surface of activated platelets.

Factor VIII is one of the lowest expressed coagulation factors in blood plasma, which is kept in the blood by binding to circulating VWF. Although FVIII does not possess a Gla-domain, it can bind to PS-exposing platelets via the C2 domain, likely in a similar manner as FV (Heemskerk et al. 2013). Once activated, FVIIIa interacts with *FIXa* on the surface of PS-exposing platelets to form the tenase complex catalyzing FXa generation. Microscopic observation and binding studies indicate that both FIXa and FXa are localized on PS-exposing platelets, while FVIIIa is detected there at a very limited amount (London et al. 2006; Swieringa et al. 2015). The majority of FVIII in a thrombus, instead, was found to co-localize with VWF that is present at

collagen and fibrin fibers. Current understanding is that once the light chain of VWF-bound FVIII is cleaved, the FVIIIa dissociates from VWF and then interacts with FIXa present on PS-exposing platelets (Swieringa et al. 2015).

Even at high TF concentrations, both FVIII and FIX are limiting factors for thrombin and fibrin formation at a thrombus independently of the flow conditions and, remarkably, also for platelet PS exposure (Swieringa et al. 2015). This points to a potent positive feedback loop of FVIIIa-/FIXa-mediated generation of FXa and thrombin, leading to enhanced thrombin-dependent PS exposure. By binding to VFW, FVIII can indirectly interact with GPIb-V-IX on platelets. However, in kinetic studies of fibrin formation, a role for VWF-bound FVIII could not be detected in platelet-dependent fibrin formation (Cosemans et al. 2011). On the other hand, it was shown that functionally active VWF readily incorporates into the fibrin network in a thrombin-dependent way (Miszta et al. 2014).

Both in vivo and in vitro studies have convincingly shown that locally generated thrombin and fibrin have a strong potentiating effect on arterial thrombus formation, thus pointing to positive feedback loops of, e.g., collagen-initiated platelet aggregation and thrombin-/fibrin-dependent consolidation and growth of the platelet aggregates (Munnix et al. 2005; Furie and Furie 2005; Dubois et al. 2007; Kuijpers et al. 2008; Stalker et al. 2014). This also explains why pharmacological inhibition of thrombin or FXa can suppress the thrombus-forming process in human and mouse blood (Kalia et al. 2008; Swieringa et al. 2015; Fukuda et al. 2011).

Coated Platelets, FXIII, and Fibrin Formation

The transglutaminase FXIII, once activated by thrombin, can cross-link multiple proteins by forming glutamyl-lysine bridges such as present in fibrin polymers and other fibrillar proteins. Active FXIII is a prerequisite for platelet clot retraction (Kasahara et al. 2010). Experiments with immobilized FXIIIa have shown that it binds to platelets via the integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (Magwenzi et al. 2011). Platelets themselves contain and secrete high levels of the catalytic subunit of FXIII (Burkhart et al. 2012), likely serving to ensure a locally high transglutaminase activity within the thrombus with activated platelets. The secretion mechanism of FXIII is not yet fully understood (Mitchell et al. 2014). In the formation of so-called coated platelets (previously COAT platelets), the transglutaminase FXIII has been implicated in the chemical cross-linking of secretory proteins derived from α-granules at the platelet surface (Dale et al. 2002). Coated platelets are invariably considered as platelets that are formed after combined stimulation of the collagen and thrombin receptors, but are often confused with PS-exposing platelets (Mattheij et al. 2016a). The original description of coated platelets refers to the formation of a covalent coat of transglutaminase-dependent serotonin conjugations of multiple secreted proteins (thrombospondin, fibrinogen, fibronectin, VWF, FV, and TFPI) at the platelet surface (Szasz and Dale 2002; Maroney et al. 2007). Tissue-type transglutaminase expressed by platelets may contribute, along with FXIII, in this cross-linking of granular proteins (Jobe et al. 2005).

Considering that coated platelets also expose PS (Alberio et al. 2000), the question arises whether the two platelet populations are identical. Dual labeling studies of thrombi with annexin A5 to probe exposed PS and a serotonin label to probe the cross-linking indicated partial but not complete overlap of the two platelet populations (Munnix et al. 2007). However, stimulation of washed platelets with a Ca^{2+} ionophore in the absence of thrombin results in PS-exposing platelets without a coat of activated FV (Alberio et al. 2000) and without high fibrin(ogen) (Jobe et al. 2005). Recently, it could be elucidated that the transglutaminase-dependent coat formation occurs secondarily to PS exposure (Mattheij et al. 2016a). The emerging concept now is that thrombin-induced FXIII activation is needed to cross-link multiple proteins to the platelet surface, among which fibrin fibers. Coated platelets therefore appear to be in control of the platelet-dependent formation of fibrin fibers, requiring both transglutaminase and integrin $\alpha_{IIb}\beta_3$ interactions (Mattheij et al. 2016a). Functionally active FXIII on the surface of PS-exposing platelets can also have an antifibrinolytic effect by incorporating α_2-antiplasmin to the fibrin coat (Mitchell et al. 2014).

The platelet- and plasma-derived FXIII pools are both essential for clot stabilization, in particular by cross-linking the single fibrin strands, which consist of high molecular weight α-fibrin polymers, which upon formation of γ-γ dimers provide strength to the fibrin network (Reed et al. 1992).

Anticoagulation Factors

The coagulation process is confined in time and place by activity of several *anti*coagulation factors, which act at different levels of the coagulation cascade. Best studied is the protein C/protein S anticoagulant system and furthermore tissue factor pathway inhibitor (TFPI) and antithrombin, which all attack and inhibit the active site of specific coagulation factors. Limited knowledge is present of other serine protease inhibitors (serpins), such as protease nexin-1.

Protein C and protein S Since the 1980s, it is known that activated protein C (APC) inactivates FVIIIa and FVa, thus suppressing the activity of the tenase and prothrombinase complexes, respectively. Protein C needs to be activated by thrombin, a process that normally occurs on endothelial cells in the vessel wall expressing thrombomodulin and the EPCR protein C receptor (Versteeg et al. 2013). The activated protein C (APC) associates with its cofactor, protein S, and then binds via Gla-domains to PS-exposing membranes to form a complex that cleaves and thereby inactivates nearby FVa and FVIIIa. Mechanistic evidence mostly comes from plasma experiments with purified phospholipid membranes. How potent the suppression of the APC/protein S complex is in platelet-dependent thrombin generation under physiological conditions still needs to be sorted out. On the other hand, it is known that a mutation in FV, designated as FV^{Leiden}, removing the APC cleavage site, results in a significantly increased risk of venous thromboembolism (Bertina et al. 1994). However, in agreement with in vivo mouse experiments of arterial thrombus formation (Kuijpers et al. 2008), this mutation does not appear to be predictive for arterial thrombosis. This may suggest that the protein C/protein S system is most effective at low shear flow conditions or stasis as present in the venous system.

TFPI is a Kunitz-type protease inhibitor that limits coagulation initiation. It is present in plasma, abundantly stored in platelets, and expressed on vascular cells (Versteeg et al. 2013). Most active is the isoform TFPIα, which contains all three Kunitz domains. Circulating at a low concentration, its level is markedly increased by secretion from platelets. The second isoform, TFPIβ, is anchored on endothelial cells and needs to be cleaved for appearance in the circulation (Girard et al. 2012). TFPI suppresses coagulation through the inhibition of TF/FVIIa as well as FXa, in the latter case in a protein S-dependent way (Hackeng et al. 2006). Since protein S can bind to PS-exposing platelets, it is likely that the protein S-TFPI complex shuts down the thrombin-generating activity of FXa on procoagulant platelets. In agreement with this notion, it has recently been demonstrated that TFPIα can inhibit prothrombinase activity (Wood et al. 2013).

Antithrombin and other serpins Antithrombin (previously antithrombin III), which belongs to the class of serpins with limited expression in platelets (Burkhart et al. 2014), is a main inhibitor of thrombin activity and generation in plasma. Its importance can be derived from the fact that it turns off three proteases, namely, FIXa, FXa, and thrombin (Versteeg et al. 2013). The activity of antithrombin is greatly enhanced by heparins (note that short-chain heparins only enhance the inactivation of FXa by antithrombin) (Li et al. 2004). There is no strong evidence yet that antithrombin massively binds to the thrombus site.

Other serpins identified in platelets are antitrypsin, with an unclear function in coagulation, and protease nexin-1 (Burkhart et al. 2014). The latter inhibits fibrinolysis by suppressing the cleavage of plasminogen and of fibrin degradation (Boulaftali et al. 2011).

Fibrinolysis Control

Platelets regulate fibrinolysis by releasing fibrinolytic and antifibrinolytic factors, including plasminogen, plasminogen activator inhibitor-1 (PAI-1), thrombin activatable fibrinolysis inhibitor (TAFI), and α_2-antiplasmin. These factors are contained in the platelet α-granules and are liberated upon activation, for instance, with thrombin (Mosnier et al. 2003; Coppinger et al. 2004). After the early evidence that fibrin formation at the platelet surface enhances the binding of plasminogen (Miles and Plow 1985), multiple receptors for plasminogen have been proposed (Miles et al. 2012). Plasminogen bound to platelets promotes fibrinolysis, is more susceptible for cleavage into plasmin (Dejouvencel et al. 2010), and is protected from the inactivation by α_2-antiplasmin (Hall et al. 1991). Recent work demonstrates two populations of platelets with bound plasminogen, i.e., platelets that bind plasminogen directly and PS-exposing platelets that bind plasminogen indirectly via platelet-bound fibrin. The latter platelet population displays enhanced capacity to generate plasmin. Strikingly, on PS-exposing platelets, plasminogen co-localizes with PAI-1, indicating that local plasminogen cleavage must exceed the inhibitory effect of PAI-1 in order to effectuate fibrinolytic activity (Whyte et al. 2015).

Novel Concept of Platelet-Controlled Coagulation

Summarizing the findings of particularly recent work, earlier models of cell-controlled coagulation (Monroe and Hoffman 2006; Versteeg et al. 2013) can now be transformed to a concept that is even more interactive than those previously considered. Initial triggering of the extrinsic coagulation system occurs by de-encrypted vascular TF (with PS-exposing membranes nearby), generating first amounts

of FVIIa, FXa, and thrombin (Fig. 1a). The thrombin activates multiple coagulation factors, among which FV and FVIII. Initiation of the intrinsic coagulation system can start by FXII binding and activation at collagen fibers or other negatively charged surfaces, with ensuing FXI activation. In a shear-dependent way, platelet will adhere to VWF/collagen (also laminin and other subendothelial matrix proteins) via GPVI and co-receptors. This same set of triggers appears to be active after injury of a healthy vessel or upon rupturing of an atherosclerotic plaque (Mastenbroek et al. 2015). Platelets experiencing a high cytosolic Ca^{2+} will expose PS, swell (balloon), and expand their procoagulant surface, whereas the majority of platelets with active integrins—forming the inner shell of a thrombus—remain aggregated and contract. Platelet secretion of multiple coagulation factors (e.g., fibrinogen, prothrombin, FV, FXIII,

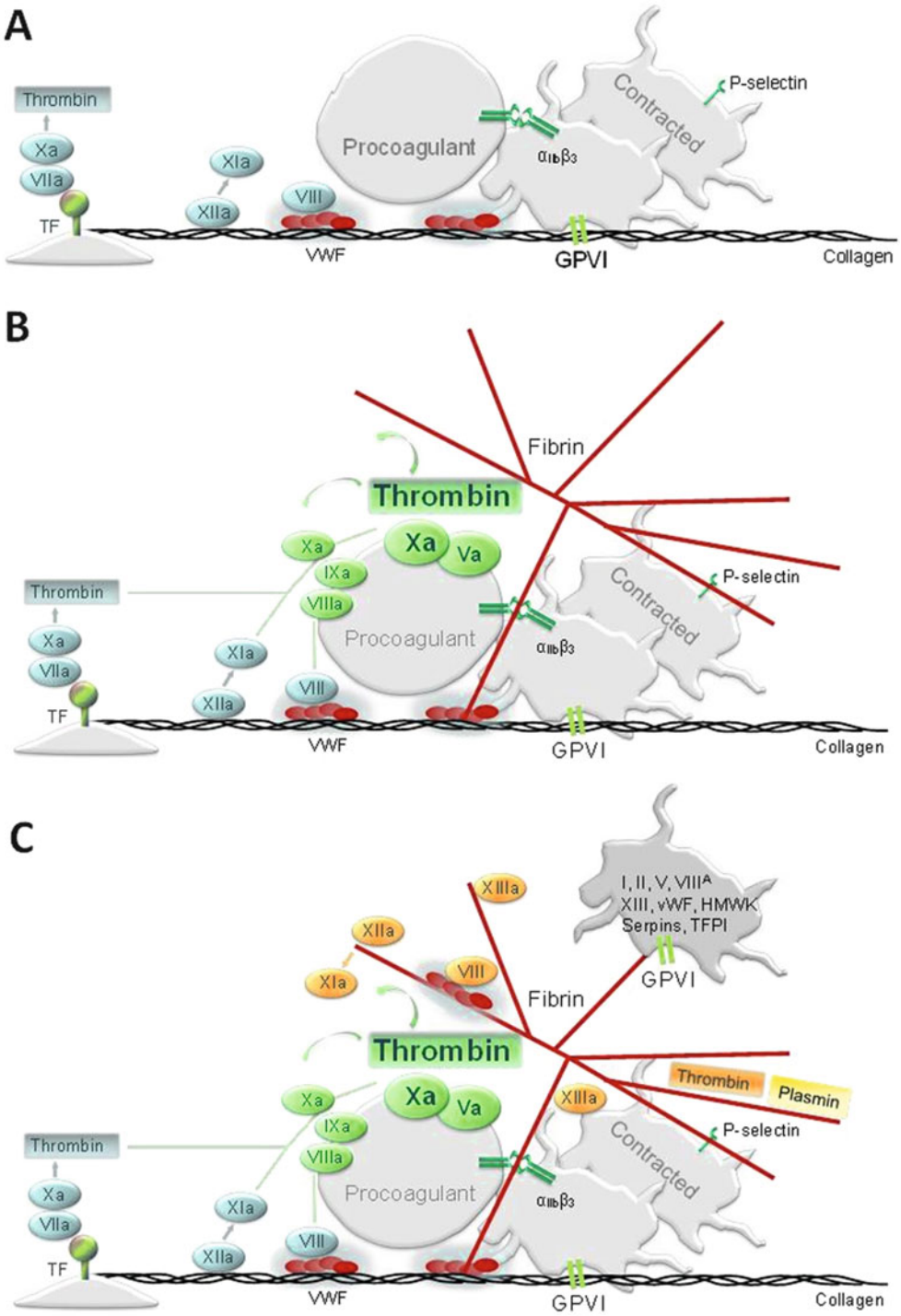

Fig. 1 Concept of staged interactions of platelet activation and coagulation in thrombus formation. (**a**) Initial activation of the extrinsic (TF) and intrinsic (FXII) coagulation pathways leading to traces of thrombin and FIXa, together with shear-dependent adhesion and activation of platelets via collagen/VWF and other adhesive proteins. (**b**) Increased generation of FXa and thrombin via procoagulant, PS-exposing membranes, leading to the formation of fibrin within a thrombus and depending on the flow conditions extending outside of the thrombus. (**c**) Fibrin fibers act as a scaffold for further platelet activation (via GPVI), coagulation factor binding (thrombin, VWF/FVIII, FXII, FXIII) further enhancing the thrombus-forming and clotting process

VWF, high molecular weight kininogen) (Burkhart et al. 2012) ensures that sufficient clotting capacity is present to start a next level of increased generation of thrombin and fibrin.

In the later stages (Fig. 1b), larger amounts of FXa and thrombin are generated via the tenase and prothrombinase complexes, respectively. FVIII bound to VWF is cleaved and will reach the sites of FX activation. Fibrin fibers are formed, heterogeneously, within and outside the platelet thrombus, but this is not an end stage, as this can act as the beginning of a new cycle of platelet activation (via GPVI), coagulation factor binding (thrombin, VWF/FVIII, FXII, FXIII), which further enhances the thrombus-forming and clotting process. Anticoagulants (protein C/S, antithrombin, TFPI), inhibiting especially FXa and thrombin, stop and confine the clotting process.

Take Home Messages

- Platelets direct and steer the coagulation process in various ways.
- Platelets interfere with the intrinsic, extrinsic, and common coagulation pathways as well as with anticoagulation processes.
- Interactions between platelets and coagulation pathways continue until later stages of fibrin thrombus formation.

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Platelets and Fibrinolysis

Mario Colucci, Nicola Semeraro, and Fabrizio Semeraro

Abstract

Platelets contain and/or bind an array of fibrinolytic components and related proteins, whereby they can both down- and upregulate the fibrinolytic process. The generally accepted role of platelets is to confer resistance to fibrinolysis in order to prevent untimely removal of the hemostatic plug. This task is accomplished mainly, but not exclusively, through clot retraction, release of huge amounts of plasminogen activator inhibitor 1, and enhancement of thrombin generation, which in turn activates a number of antifibrinolytic pathways. Recent findings, however, suggest that the profibrinolytic properties of platelets also participate in the modulation of fibrinolysis, probably at later stages when the process enters the lytic phase. Moreover, in a reciprocal fashion, the activation of fibrinolysis could have profound and paradoxical effects on platelet function. This chapter outlines current knowledge on the complex cross-talk between platelets and fibrinolysis and its implications for physiological and pathological hemostasis.

Introduction

Platelets have been known for more than 50 years to take part in the modulation of fibrinolysis. Since publication of the first papers on the subject, it has been clear that the effects of platelets on fibrinolysis are complex and that platelets are able to both down- and upregulate fibrinolysis (Johnson and Schneider 1953; Stefanini and Murphy 1956; Greig and Cornelius 1961; Reid and Silver 1964). Since then, our knowledge of the biology of the fibrinolytic system, its regulatory mechanisms, and its multiple interactions with platelets and other intravascular and extravascular cells has dramatically improved. Available evidence now indicates that platelets contain and/or bind an array of fibrinolytic components and related proteins, whereby they exert both antifibrinolytic and profibrinolytic effects. In a reciprocal fashion, activation of fibrinolysis can have profound effects on platelet function (Coller 1990; Pasche and Loscalzo 1991). How these manifold phenomena influence the fibrinolytic process during physiological hemostasis and thrombosis is still debated and a matter of active investigation. From literature data, it appears that platelets are major players in conferring fibrinolytic resistance to blood clots or thrombi, particularly platelet-rich arterial thrombi. However, there is increasing awareness that thrombi formed in vivo contain different platelet populations, which could play different roles at different times (Heemskerk et al. 2013; Ivanciu and Stalker 2015), suggesting that the profibrinolytic properties of platelets also participate in the modulation of fibrinolysis.

This chapter outlines current knowledge on the anti- and profibrinolytic properties of platelets, how they affect fibrin removal during hemostasis and thrombosis, and how fibrinolytic enzymes modify platelet function. Before considering these aspects, we briefly review the intravascular fibrinolytic system and its main regulatory mechanisms.

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The Fibrinolytic System and Its Regulation

Within the vascular system, fibrinolysis fulfils two major functions: (1) it removes the provisional matrix created by the hemostatic process following vascular injury and (2) prevents or limits fibrin deposition at sites of aberrant clotting activation, thereby preserving vascular integrity.

The central reaction of the fibrinolytic process is activation of plasminogen to form plasmin, a trypsin-like protease that degrades the fibrin network into soluble fragments (fibrin degradation products; FDP) (reviewed by Collen 1999). Plasminogen activation is catalyzed by two distinct enzymes, tissue plasminogen activator (t-PA) and urokinase-type PA (u-PA). The fibrinolytic enzymes are controlled by specific inhibitors, namely plasminogen activator inhibitor-1 (PAI-1), which inactivates the plasminogen activators, and α_2-antiplasmin (α_2-AP), a fast-acting plasmin inhibitor. PAI-2 is another inhibitor of plasminogen activators, but it is undetectable in plasma, except during pregnancy and in some pathological conditions (Medcalf and Stasinopoulos 2005).

The t-PA-mediated pathway is primarily involved in intravascular fibrinolysis, whereas the u-PA pathway mainly participates in phenomena such as cell migration and tissue remodeling (Murphy et al. 1992). The activation of plasminogen is a localized phenomenon and takes place on fibrin and cell surfaces through binding to specific receptors for plasminogen activators and plasminogen (Miles and Parmer 2013). The contact system of coagulation has also been implicated in fibrinolysis through direct activation of plasminogen by factor XIIa, which is structurally related to t-PA and u-PA (Goldsmith et al. 1978; Tans and Rosing 1987), and through the kallikrein-mediated conversion of the inactive single-chain u-PA (scu-PA) to the active two-chain form (Ichinose et al. 1986).

Regulation of the fibrinolytic process is complex and involves several mechanisms, which include activation of zymogens, specific inhibition of active enzymes, regulation of circulating levels of fibrinolytic factors, and interaction with fibrin and cell surfaces. The process is modulated by numerous factors in addition to the main components of the fibrinolytic system, some of which are relevant to the present chapter and are discussed in later sections. Here, we describe the main regulatory mechanisms, namely the balance between t-PA and PAI-1, interaction with fibrin, and coagulation–fibrinolysis cross-talk.

The serine protease t-PA is primarily involved in the dissolution of fibrin in the circulation, and thus its availability in free form is of utmost importance for efficient fibrinolysis. Circulating t-PA is released by the endothelium as an enzymatically active single-chain molecule, in contrast to the single-chain precursor form of most serine proteases (Rijken and Lijnen 2009). Cleavage by plasmin converts single-chain t-PA into a two-chain form, whose functional properties do not differ substantially from its precursor. The plasma concentration of t-PA is in the picomolar range, with significant circadian variations (Angleton et al. 1989). PAI-1 derives from different sources, including liver, endothelial cells, macrophages, and adipocytes (reviewed by Dellas and Loskutoff 2005). It is contained in platelets in considerable amounts, as discussed below. The origin of circulating PAI-1 remains to be fully defined and could vary depending on the health status (e.g., body weight) and type of disease (Tjärnlund-Wolf et al. 2012). PAI-1 is a very fast inhibitor of t-PA, the second-order rate constant of the reaction being in the order of 10^7 M^{-1} s^{-1}. The normal plasma concentration of PAI-1 is in the picomolar range, but is generally higher than that of t-PA, meaning that most circulating t-PA is in complex with PAI-1. The half-life of both t-PA and PAI-1 is about 5 min, which implies a continuous release of these proteins into the circulation to maintain their plasma concentrations. In striking contrast with these two factors, plasminogen and α_2-AP (both of liver origin) are present in plasma in much higher concentrations (micromolar range) and have plasma half-lives of the order of days. Thus, t-PA and PAI-1 represent the dynamic part of the system and any modification in the synthesis and release of t-PA and/or PAI-1 results in a sudden change in fibrinolytic capacity. The rate of synthesis and/or release of t-PA by endothelial cells is increased by a number of substances, including thrombin, histamine, tumor necrosis factor α (TNF-α), and norepinephrine, and by stress conditions such as venous occlusion and exercise (reviewed by Oliver et al. 2005). The regulation of PAI-1 is achieved primarily by alterations in the rate of gene expression. Inducers of PAI-1 synthesis include TNF-α, interleukin 1 (IL-1), transforming growth factor β (TGF-β), thrombin, insulin, angiotensin II, and corticosteroids (Dellas and Loskutoff 2005).

Fibrin has a central and very important role in the regulation of fibrinolysis. First of all, fibrin is a cofactor for t-PA-mediated plasminogen activation. In solution, in the absence of fibrin or fibrin-derived products, t-PA is a poor activator of plasminogen and no appreciable plasmin is formed in the circulation, even when the levels of free t-PA increase manyfold following strenuous exercise or venous occlusion. When a clot is formed, both t-PA and plasminogen bind to fibrin, forming a ternary complex that dramatically reduces the Km for plasminogen activation so that plasmin is efficiently generated at its site of action. Binding of t-PA to fibrin is mediated by the finger domain and the second kringle domain, whereas binding of plasminogen is mediated by the lysine binding sites located in the kringle domains, which are also essential for the interaction of plasmin with α_2-AP (Rijken and Lijnen 2009). During fibrin degradation by plasmin, new binding sites are exposed, identified as C-terminal lysines, which enhance the

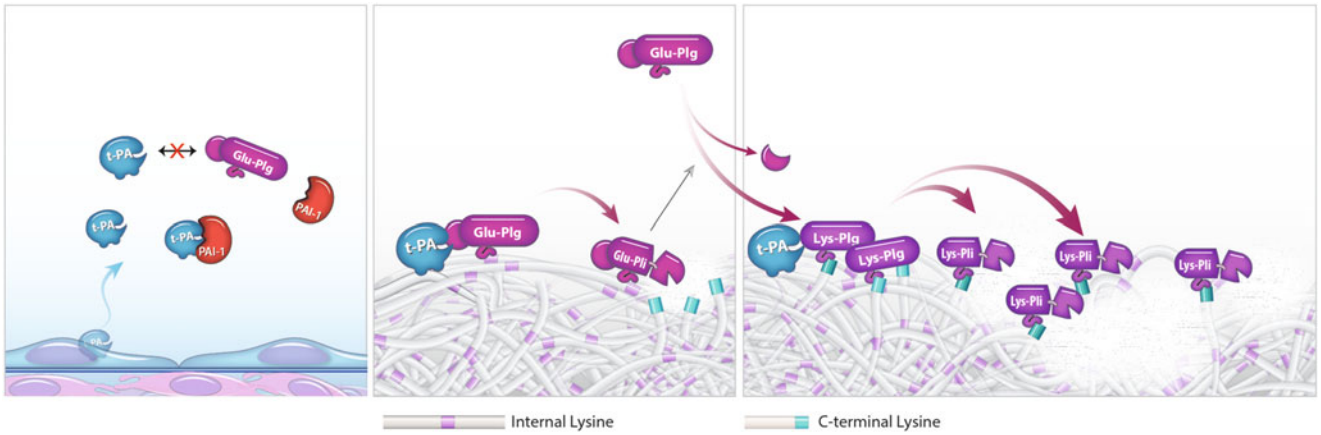

Fig. 1 Role of fibrin as cofactor in tissue-type plasminogen activator (t-PA)-mediated plasminogen activation. The t-PA released from endothelial cells is an inefficient activator of plasminogen (Plg) in solution and is quickly inhibited by plasminogen activator inhibitor 1 (PAI-1) (*left*). In the presence of a fibrin clot, both t-PA and plasminogen bind to fibrin forming a ternary complex in which the activity of t-PA towards plasminogen is highly augmented and the first molecules of plasmin (Pli) are generated (*middle*). After this initial slow phase, plasmin upregulates its own production in two ways (*right*): (1) by degrading fibrin, it exposes new binding sites (C-terminal lysines), which dramatically increase the accumulation of plasminogen on fibrin; and (2) it converts Glu-plasminogen to Lys-plasminogen, a more readily activatable form of plasminogen

accumulation of plasminogen on the clot surface up to 30-fold and increase the lysis rate. Moreover, native plasminogen, which has N-terminal glutamic acid (Glu-plasminogen), is converted by limited plasmic digestion to Lys-plasminogen. This has a more open conformation and is therefore a better substrate for plasminogen activators, further enhancing plasmin generation and fibrin degradation (Fig. 1). Another important consequence of the binding of fibrinolytic enzymes to fibrin is that they are protected from their respective inhibitors. Indeed, fibrin-bound t-PA is neutralized more slowly by PAI-1 (Kruithof et al. 1984; Chmielewska et al. 1988; Thelwell and Longstaff 2007) and the rate of plasmin inhibition by α_2-AP is 100- to 1000-fold lower if the enzyme is bound to fibrin (Rijken and Lijnen 2009). In addition, t-PA (either plasma-derived or released locally) could accumulate on fibrin and reach concentrations severalfold higher than the circulating levels (Tran-Thang et al. 1984).

Fibrin also modulates the function of fibrinolytic inhibitors. PAI-1 binds reversibly to fibrin and maintains its inhibitory activity (Wagner et al. 1989; Reilly and Hutzelmann 1992; Podor et al. 2000) and α_2-AP is crosslinked to fibrin by factor XIIIa (FXIIIa) (Sakata and Aoki 1980). Compelling evidence indicates that fibrin-bound inhibitors play a major role in restraining the lytic process through inhibition of target enzymes within the clot (Sakata and Aoki 1982; Reed et al. 1990; Podor et al. 2000). These multiple interactions emphasize the multifaceted role of fibrin, which can both down- and upregulate its own degradation. These opposite functions are important for fine-tuning the fibrinolytic process and are likely to take place at different moments in order to impede untimely removal of the hemostatic plug.

The extent and the dynamics of clotting activation have a great impact on the fibrinolytic process (reviewed by Nesheim 2003; Wolberg 2007). Thrombin generation, in particular, contributes to fibrinolytic resistance through different mechanisms, which are discussed in more detail in later sections. Suffice to say here that these mechanisms include (1) a direct effect of thrombin concentration on fibrin structure, which in turn determines clot stability and resistance to proteolytic degradation (Blombäck et al. 1994); (2) activation of FXIII, a transglutaminase that introduces covalent bonds into fibrin and crosslinks several proteins to fibrin, including α_2-AP, which is one of the most important fibrin-associated fibrinolysis inhibitors (Muszbek et al. 2011); and (3) activation of TAFI (thrombin-activatable fibrinolysis inhibitor), a carboxypeptidase that inhibits plasminogen activation on the fibrin surface (Wang et al. 1998). The close functional link between coagulation and fibrinolysis has important implications and suggests that fibrinolytic alterations could be much more frequent than previously thought. Numerous studies indicate that conditions of hypo- or hypercoagulation are accompanied by decreased or increased fibrinolytic resistance, respectively. A typical example is hemophilia (A or B), which is characterized by the formation of clots that are highly susceptible to fibrinolysis (Broze and Higuchi 1996; Mosnier et al. 2001). The phenomenon can be corrected by replenishing the missing factor or supplementing plasma with supraphysiological levels of antifibrinolytic factors (e.g., TAFI or FXIII) (Broze and Higuchi 1996; Mosnier et al. 2001; Rea et al. 2014). Similar fibrinolytic changes have been detected in patients under anticoagulant treatment (Incampo et al. 2013), even though not all anticoagulant drugs are equally effective as profibrinolytic agents at clinically relevant

concentrations (Semeraro et al. 2016). On the other hand, patients with heightened thrombin generation, such as carriers of thrombophilic mutations (Colucci et al. 2004; Ammollo et al. 2014) or prothrombotic factors (Colucci et al. 2003), display increased in vitro fibrinolytic resistance, which correlates fairly well with thrombin generation capacity. This concept can be extended to many conditions associated with increased risk of thrombosis, where the fibrinolysis impairment caused by aberrant thrombin formation could contribute to the thrombotic tendency.

The importance of fibrinolysis in physiological hemostasis is underscored by the clinical phenotype of subjects lacking factors involved in regulation of the fibrinolytic process. Patients with congenital deficiency in α_2-AP, PAI-1, or FXIII exhibit a tendency to moderate to severe bleeding characterized by delayed hemorrhage after trauma or surgical procedures (Carpenter and Mathew 2008; Mehta and Shapiro 2008; Biswas et al. 2014), which typically reflects the premature lysis of highly susceptible hemostatic plugs. Conversely, disturbance of fibrinolysis regulation leading to increased fibrinolytic resistance could result in predisposition to thrombosis. Indeed, clinical studies have documented increased levels of PAI-1 in association with arterial thrombosis (Hamsten et al. 1985) and microvascular occlusion in disseminated intravascular coagulation (Hack 2001). Moreover, elevated TAFI levels have been suggested to represent a risk factor for venous thromboembolism and ischemic stroke (Heylen et al. 2011). Augmented risk of venous and arterial thrombosis has also been found in subjects with overall hypofibrinolysis (Lisman et al. 2005; Guimaraes et al. 2009), as assessed by global in vitro assays that depict the complex interplay between fibrinolytic and coagulation pathways. The pivotal role of fibrinolysis is further highlighted by the widespread use of antifibrinolytic drugs such as the lysine analogs ε-aminocaproic acid (EACA) and tranexamic acid, and the serine protease inhibitor aprotinin (whose use is limited because of safety concerns) (De Hert et al. 2015) to reduce bleeding and transfusion needs in hemorrhagic surgical and medical conditions such as cardiac surgery, liver transplantation, hemophilia (Sindet-Pedersen and Stenbjerg 1986; Casati et al. 1999; Hvas et al. 2007; Molenaar et al. 2007), and, of particular relevance in the context of this chapter, thrombocytopenia (Kalmadi et al. 2006).

Platelet Antifibrinolytic Properties

The main features of fibrinolytic inhibitors and antifibrinolytic functions associated with platelets are summarized in Table 1.

Table 1 Main characteristics of platelet-associated fibrinolytic inhibitors

Platelet factor or function	Localization	Main function(s)	Concentration relative to the plasma pool (%)	Additional information
Clot retraction	Mediated by motility proteins and $\alpha_{IIb}\beta_3$	Decreases clot permeability, alters fibrin structure, reduces t-PA binding to fibrin, increases resistance to plasmin	–	Major mechanism of fibrinolytic resistance
PAI-1	α-Granules	Binds fibrin through vitronectin Fast inhibitor of t-PA	900	Largely inactive Major mechanism of fibrinolytic resistance
TAFI	α-Granules	TAFIa removes plasminogen binding sites from fibrin	0.1	Besides releasing TAFI, platelets enhance TAFI activation by promoting thrombin formation
FXIII	Cytoplasm	Fibrin crosslinking Crosslinking of α_2-AP and other factors to fibrin	50	Consists of A subunits only (FXIII-A_2) Protease-independent activation
PolyP	Dense granules	Clotting activation Alteration of fibrin structure	Undetectable in plasma	Enhances thrombin-mediated activation of TAFI
α_2-AP	α-Granules	Fast inhibitor of plasmin	0.05	Fibrin crosslinked α_2-AP plays a major role in fibrinolytic resistance
PN-1	α-Granules Platelet surface	Inhibits fibrin-bound t-PA and fibrin-bound plasmin	Undetectable in plasma	Complementary role with PAI-1 and α_2-AP
TFPI-2	Probably α-granules	Inhibits plasmin	Very low plasma levels	Inhibits the contact pathway
Zinc	Cytoplasm α-granules	Inhibits plasminogen activation and plasmin activity	Variable (≈50)	Most plasma zinc is bound to proteins
Platelet-derived phospholipids	Membranes	Diffusion barrier; reduces the binding of fibrinolytic enzymes to fibrin, increases resistance to plasmin	–	Loss of platelet phospholipids after strong activation or death

PAI-1 plasminogen activator inhibitor 1, *TAFI* thrombin activatable fibrinolysis inhibitor, *FXIII* factor XIII, *PolyP* polyphosphate, *α_2-AP* α_2-antiplasmin, *PN-1* protease nexin 1, *TFPI-2* tissue factor pathway inhibitor 2

Clot Retraction and Clot Structure

Platelets contract the blood clot through the action of cytoplasmic motility proteins, such that fluid is expelled. This process is called clot contraction or retraction and is believed to reinforce hemostasis by forming a seal, and restores blood flow by decreasing the area obstructed by intravascular clots (Carr 2003; Muthard and Diamond 2012). Clot retraction is dependent on fibrin(ogen) binding to active $\alpha_{IIb}\beta_3$ integrin (glycoprotein IIb/IIIa; GPIIb/IIIa) on the platelet surface and transmits the contractile forces generated in the platelet actin–myosin cytoskeleton to connecting fibrin fibers around platelets (Schoenwaelder et al. 2010), thereby transforming a diffuse fibrin network into a small and dense platelet–fibrin clot. The process results in clot stiffening, which is dependent both on direct reinforcement by platelets and on strain stiffening of fibrin fibers under tension as a result of platelet contraction (Lam et al. 2011).

Clot retraction is an important mechanism through which platelets reduce the lysability of clots. If platelet-rich clots are formed in the presence of inhibitors of platelet contraction (e.g., cytochalasin D) or antagonists of the $\alpha_{IIb}\beta_3$ receptor (monoclonal antibodies or the ligand-mimetic peptide D-RGDW), clot retraction is prevented and fibrinolysis is markedly hastened (Kunitada et al. 1992; Collet et al. 2002; Carrieri et al. 2011). In contrast to these findings, a few studies have reported accelerated fibrinolysis associated with clot retraction (Carroll et al. 1981, 1982; Hoki et al. 2009). The latter studies, however, were performed in rather unphysiological conditions, such as with extensive dilution of blood or platelet-rich plasma or use of the euglobulin fraction instead of plasma. The mechanisms behind fibrinolytic resistance induced by clot retraction are complex and not fully understood. Clot compression causes the extrusion of serum from the clot, thereby reducing the amount of plasminogen available within the fibrin mesh, a phenomenon that can be reproduced by mechanical compression of a plasma clot devoid of platelets (Sabovic et al. 1989). In addition, the low permeability of the retracted clot hinders the transport of proteins from the outside. Another important consequence of clot retraction is the reduction in t-PA binding to fibrin, which, as noted above, is an essential step for efficient fibrinolysis. Kunitada et al. (1992) reported that platelet-rich plasma clots bound some 60 % less t-PA than platelet-poor plasma clots. Importantly, the reduction in t-PA binding was observed over a wide range of t-PA concentrations, including pharmacological concetrations, and was paralleled by a reduction in clot lysis. By contrast, t-PA binding to the platelet-rich clot is not impaired if clot retraction is prevented by either cytochalasin D or anti-$\alpha_{IIb}\beta_3$ antibody (Kunitada et al. 1992). Microscopic examination revealed that platelet-rich clots are heterogeneous structures with branching fibers alternating with dense platelet-rich areas, which are made of highly compacted fibrin fibers retracted by the platelet contractile force (Collet et al. 2001). The reduction in t-PA binding and the resistance to lysis is seen around these platelet-rich areas (Collet et al. 2002), probably because of the limited access of t-PA to the compact fibrin network surrounding platelet aggregates. In addition, the stretching of fibrin fibers attached to contracted platelets might contribute to fibrinolytic resistance, as suggested by Varjú et al. (2011), who showed that mechanical stretching of fibrin impaired plasminogen activation and made the denser fibrin network less sensitive to plasmin. A schematic representation of the changes induced by clot retraction is illustrated in Fig. 2.

Concerning in vivo evidence, studies in animal models showed that integrin $\alpha_{IIb}\beta_3$ inhibitors accelerate thrombolysis and prevent reocclusion in experimentally produced coronary thromboses treated with recombinant t-PA (alteplase) (Jang et al. 1989; Yasuda et al. 1988, 1990; Kohmura et al. 1993). Moreover, a number of studies in patients with acute myocardial infarction or ischemic stroke showed that the combination of fibrinolytic agents with anti-$\alpha_{IIb}\beta_3$ accelerated recanalization and reduced reocclusion (Antman et al. 1999; Ouriel et al. 2004; Seitz et al. 2004; Straub et al. 2004; Adams et al. 2008). Furthermore, patients with Glanzmann thrombasthenia (GT), whose platelets lack $\alpha_{IIb}\beta_3$ integrin and do not support clot retraction, display great susceptibility to t-PA in an in vitro thromboelastographic model of clot lysis (Shenkman et al. 2012). However, whether and to what extent the reduced fibrinolytic resistance contributes to bleeding in GT is difficult to establish. For these patients, the clinical manifestations (typically, mucocutaneous hemorrhages with early onset after trauma), suggest that the impairment of platelet plug formation caused by the absence or dysfunction of $\alpha_{IIb}\beta_3$ is the main cause of bleeding. Nevertheless, the successful use of antifibrinolytic agents, either alone or in combination with platelet concentrates or recombinant factor VIIa, for the treatment of non-surgical and surgical bleeding in GT (reviewed by Poon et al. 2016) offers some hints about the possible importance of accelerated fibrinolysis in this disease.

In addition to clot retraction, platelets can influence clot architecture by virtue of their procoagulant function, as the concentration of thrombin at the time of fibrin assembly has a great impact on clot structure (Blombäck et al. 1994). Activated platelets play a major role in the propagation phase of coagulation by providing the most efficient surface for the assembly of coagulation factor complexes that lead to thrombin generation (Monroe et al. 2002). Fibrin clots formed in the presence of low thrombin consist of thick fibers and are highly susceptible to fibrinolysis; by contrast, clots generated in the presence of high thrombin concentrations are made up of thinner,

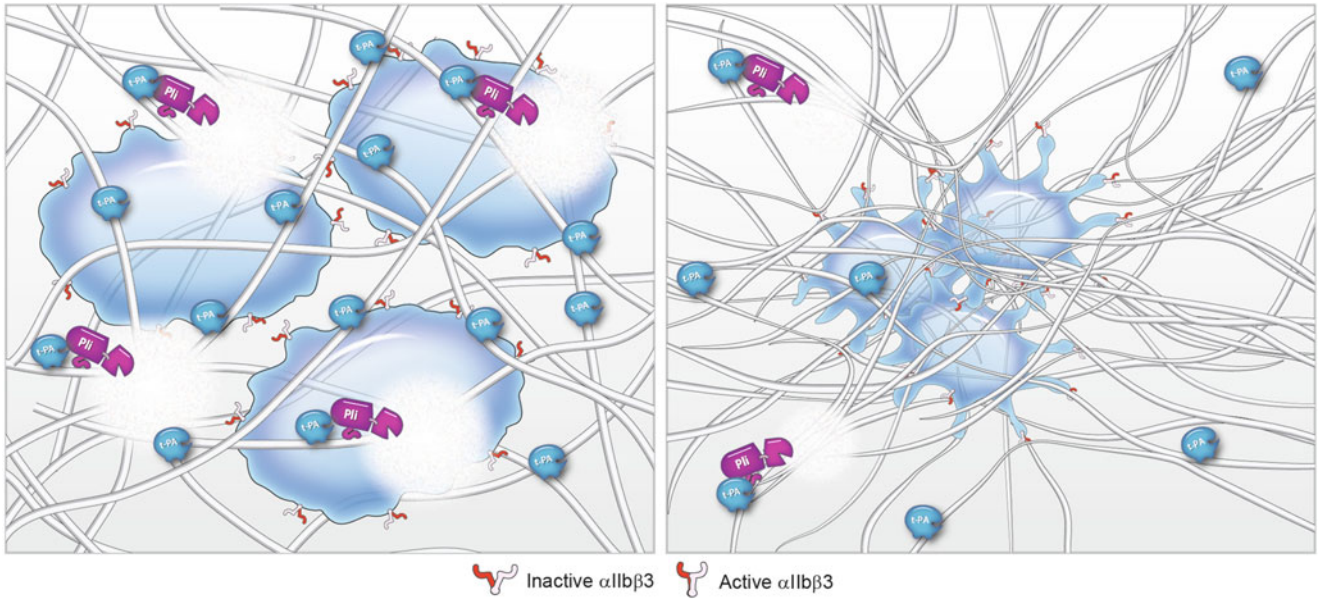

Fig. 2 Fibrinolytic resistance induced by platelet-mediated clot retraction. In the absence of clot retraction (e.g., in platelet-poor areas or when platelet contraction is prevented by platelet inhibitors), fibrin fibers are randomly oriented and organized in a homogeneous network; thus, t-PA-mediated activation of plasminogen on the fibrin surface and consequent fibrin breakdown can proceed at high speed (*left*). Areas enriched in aggregated and contracting platelets are made of fibrin fibers, which are highly compacted and stress-stiffened by the platelet contractile force. In these areas, diffusion of fibrinolytic enzymes, their binding to fibrin, activation of plasminogen, and the susceptibility to plasmin are reduced so that fibrinolysis is greatly impaired (*right*)

more tightly packed fibrin filaments that are resistant to lysis. In analogy with stretched fibrin, thin fibrin fibers support a slower rate of t-PA-mediated plasmin generation than thick fibers, reducing the overall fibrinolytic activity of the system. Moreover, clots with thin fibrin fibers are more resistant to plasmin degradation than clots with thick fibers (Wolberg 2007).

Thrombin-mediated improvement of fibrin structure might be predominant in thrombus areas occupied by procoagulant, non-contracting platelets, which form a distinct platelet population characterized by surface-exposed phosphatidylserine (PS) (Heemskerk et al. 2013). The importance of procoagulant platelet phospholipids in the formation of fibrinolysis-resistant fibrin gel structure has been documented in vitro in platelet-rich plasma clots from both normal subjects and patients with GT (He et al. 2005). On theoretical grounds, the formation of clots with inadequate structure can occur in patients with Scott syndrome, a rare bleeding disorder characterized by a defect in membrane lipid scrambling (Zwaal et al. 2004). The platelets of these patients are unable to expose the procoagulant phospholipids (particularly PS) on the outer membrane upon activation, and thus do not support tenase and prothrombinase assembly and, in turn, thrombin generation. To date, however, there is no formal evidence that platelets carrying this defect do not support the formation of a lysis-resistant fibrin clot.

Plasminogen Activator Inhibitor 1

Plasminogen activator inhibitor 1 (PAI-1) is a member of the SERPIN superfamily and is the principal inhibitor of the plasminogen activators t-PA and u-PA. It binds irreversibly to target enzymes, giving rise to an inactive complex (reviewed by Dellas and Loskutoff 2005). Because of a conformational flexibility, PAI-1 is unstable and spontaneously converts to an inactive (latent) form with a half-life of 1–2 h at 37 °C. Latent PAI-1 can be reactivated in vitro by denaturants such as sodium dodecyl sulfate, guanidine HCl, or urea and by negatively charged phospholipids (Lambers et al. 1987), a finding that led to the hypothesis that latent PAI-1 could be reactivated on the surface of activated platelets. It is unknown, however, whether reactivation of PAI-1 takes place in vivo. Another important feature of PAI-1 is that it binds fibrin without losing its capacity to neutralize t-PA (Wagner et al. 1989). Even though PAI-1 can directly associate with fibrin through low-affinity binding sites, the physiologically relevant PAI-1–fibrin interaction is mediated by vitronectin, which serves as an intermolecular bridge to support high-affinity binding of PAI-1 to fibrin. Furthermore, vitronectin-mediated binding to fibrin stabilizes PAI-1 and enhances its inhibitory effect on t-PA-induced clot lysis (Podor et al. 2000).

In blood, two different pools of PAI-1 exist, plasma and platelet pools. Platelet PAI-1 derives from endogenous

synthesis in megakaryocytes (Alessi et al. 1994; Hill et al. 1996) and is stored in α-granules, from where it can be released after platelet activation with thrombin (Erickson et al. 1984). Platelet PAI-1 accounts for more than 90 % of total PAI-1 in blood (Booth et al. 1988); however, because of thermal instability, a large proportion of PAI-1 is in the inactive latent form. In earlier studies, it was estimated that more than 90 % of platelet PAI-1 was in an inactive conformation (Kruithof et al. 1987; Booth et al. 1988). However, using an approach that minimizes the in vitro inactivation of PAI-1, it was recently found that more than 50 % of PAI-1 in platelets is in the active form (Brogren et al. 2011). This high percentage of active inhibitor can be explained by the ability of platelets to synthesize PAI-1 (Brogren et al. 2004) and to stabilize it through packaging with other α-granule proteins in a calcium-dependent manner (Lang and Schleef 1996). Whatever the specific activity of platelet PAI-1, platelets represent a huge reservoir of PAI-1, independent of the plasma pool. It has been calculated that the local concentration of PAI-1 at sites of platelet aggregates is more than three orders of magnitude greater than the plasma concentration (Kruithof et al. 1987; Biemond et al. 1995).

The role of platelet PAI-1 in conveying fibrinolytic resistance to clots or thrombi has been extensively investigated. In vitro, in the absence of plasma, the release of PAI-1 from stimulated platelets or the addition of a platelet lysate greatly delays fibrinolysis induced by exogenous t-PA or u-PA (Levi et al. 1992; Braaten et al. 1993; Robbie et al. 1993) but not by a PAI-1-resistant plasminogen activator (Fay et al. 1994). Under similar conditions, PAI-1-deficient platelets exhibit a diminished capacity to inhibit clot lysis (Fay et al. 1994). In the presence of plasma, the effect of platelet PAI-1 is greatly influenced by the experimental conditions. The antifibrinolytic effect of platelet PAI-1 is negligible at the normal circulating ratio of plasma to platelets; it becomes significant only when the platelet concentration is increased about 20-fold (Robbie et al. 1993). In model thrombi generated under flow conditions (Chandler loop) (Chandler 1958), platelet PAI-1 is one of the main determinants of fibrinolytic resistance (Torr-Brown and Sobel 1993; Stringer et al. 1994; Mutch et al. 2007). Chandler thrombi consist of a platelet-rich "head" and an erythrocyte-rich "tail," resembling the polarized cell distribution of arterial thrombi (Robbie et al. 1997). The head contains larger amounts of PAI-1 than the tail and is more resistant to lysis (Stringer et al. 1994). Depending on the model used, platelet-mediated fibrinolytic resistance is abolished or greatly reduced by neutralizing anti-PAI-1 antibodies (Braaten et al. 1993; Torr-Brown and Sobel 1993; Stringer et al. 1994; Mutch et al. 2007) or when a PAI-1-resistant plasminogen activator is used as thrombolytic agent (Fay et al. 1994).

In vivo experiments in animal models provide compelling evidence that PAI-1 can influence thrombus formation and growth, reocclusion, and response to thrombolytic therapy (Reilly et al. 1991a, b; Biemond et al. 1995; Friederich et al. 1997; Farrehi et al. 1998; Zhu et al. 1999). However, the role of PAI-1 in each of these phenomena cannot be generalized and depends on specific conditions, such as thrombus localization and composition, thrombotic challenge, and animal species. Platelet PAI-1 is believed to be principally involved in arterial thrombosis, because of the high platelet content of such thrombi. However, the precise contribution of platelet PAI-1 in conferring fibrinolytic resistance in vivo is difficult to define and is largely inferred from indirect evidence. Jang et al. (1989) showed that platelet-rich thrombi are more resistant to t-PA treatment than erythrocyte-rich thrombi formed in the very same femoral artery of rabbits. That platelet PAI-1 is, at least partly, responsible for the thrombolytic resistance of platelet-rich thrombi is suggested by the finding that, in a murine model of carotid artery thrombosis, reperfusion after t-PA infusion occured in PAI-deficient mice but not in wild-type animals (Zhu et al. 1999). Notably, the levels of circulating free t-PA after the thrombolytic treatment were similarly high in PAI-$1^{-/-}$ and PAI-$1^{+/+}$ mice, indicating that an important contribution of plasma PAI-1 to fibrinolytic resistance is unlikely. By contrast, neutralization of PAI-1 by a monoclonal antibody did not improve t-PA-mediated thrombolysis of erythrocyte-rich jugular vein thrombi (Biemond et al. 1995), suggesting that only platelet-rich thrombi contain enough PAI-1 to inhibit pharmacological concentrations of t-PA. In a carotid artery thrombosis model in mice, Farrehi et al. (1998) showed that residual thrombi, harvested 24 h after vessel injury, were smaller in PAI-$1^{-/-}$ mice than in wild-type mice, suggesting that platelet PAI-1 also influences spontaneous thrombolysis.

Additional support for the importance of platelet PAI-1 in fibrinolytic resistance comes from immunohistochemical analysis of human thrombi, which, in line with in vitro findings, showed that arterial thrombi contain greater amounts of PAI-1 than venous thrombi (Potter van Loon et al. 1992, Robbie et al. 1996). PAI-1 was localized in platelet-rich areas in association with fibrin strands (Robbie et al. 1997) and was shown to correlate inversely with ex vivo spontaneous lysis (Potter van Loon et al. 1992). Conceivably, thrombus PAI-1 derives from the platelets recruited during thrombus formation. However, some might have been taken up from plasma or derive from the vessel wall, because both endothelial and smooth muscle cells are able to synthesize PAI-1 (Dellas and Loskutoff 2005). Moreover, platelet-derived products (e.g., TGF-β) might stimulate PAI-1 synthesis by vascular cells, as suggested by in vitro and in vivo studies (Fujii and Sobel 1990; Slivka and Loskutoff 1991).

Studies in humans have focused mainly on hereditary PAI-1 deficiency and on acquired PAI-1 changes associated

with diverse pathological conditions. PAI-1 deficiency is very rare and only a few cases have been described so far (Schleef et al. 1989; Diéval et al. 1991; Lee et al. 1993; Fay et al. 1992; 1997; Iwaki et al. 2011). In two of these studies, the mutated PAI-1 gene was characterized and shown to harbor a frame-shift mutation that results in a new stop codon (Fay et al. 1997; Iwaki et al. 2011). Homozygous carriers of this null mutation had no detectable PAI-1 in plasma nor in platelets and had a life-long history of delayed, sometimes life-threatening, bleeding in response to trauma or surgery, which could be efficiently treated with antifibrinolytic drugs such as tranexamic acid and EACA (Fay et al. 1997). Another patient with very low PAI-1 in plasma and platelets was described by Schleef et al. (1989). This patient too had life-long bleeding problems, often requiring multiple transfusions with packed red blood cells. Two other studies reported patients with low plasma levels of PAI-1 but normal or nearly normal platelet PAI-1 (Diéval et al. 1991; Lee et al. 1993). These patients experienced recurrent bleeding episodes after trauma or surgery, which, however, tended to stop spontaneously and did not require transfusions (Diéval et al. 1991). Although the small number of known cases of PAI-1 deficiency calls for caution, it is tempting to speculate that the absence of both platelet and plasma PAI-1 is associated with more severe bleeding than the lack of plasma PAI-1 only.

Low levels of intraplatelet PAI-1 are encountered in patients with Gray platelet syndrome (GPS) (Booth et al. 1988; Vermylen et al. 1991), an inherited mild-to-moderate bleeding disorder characterized by lack of platelet α-granules and their content (Nurden and Nurden 2014). However, considering the huge number of biologically active proteins stored in α-granules, it is difficult to establish the relationship between deficiency of platelet PAI-1 and bleeding in GPS. Moreover, no study has tested whether GPS platelets differ from normal platelets in in vitro models of clot lysis.

Increased levels of PAI-1 have been reported in patients with venous and arterial thrombosis and with a number of other conditions associated with increased thrombotic risk, such as sepsis, obesity, metabolic syndrome, and diabetes (Alessi and Juhan-Vague 2006; Semeraro et al. 2012; Van De Craen et al. 2012). In the vast majority of these studies, however, only plasma PAI-1 levels and PAI-1 gene polymorphism were examined. The few reports on platelet PAI-1 in disease states indicate that the platelet pool varies independently from the plasma pool, and that platelet PAI-1 concentrations fall within the normal range in several pathological conditions in which plasma PAI-1 is generally increased (Simpson et al. 1990, 1997). Not surprisingly, the platelet pool of PAI-1 is markedly increased in patients with polycythemia vera (PV) and essential thrombocythemia (ET). In such patients, blood PAI-1 content correlates with the platelet number and with fibrinolytic resistance (Bazzan et al. 1993; Pósán et al. 1998). Interestingly, the blood of patients with PV or ET releases more PAI-1 than control blood when exposed to mild mechanical stress (Pósán et al. 1998). Moreover, patients with ET and thrombotic complications were reported to have greater amounts of intraplatelet PAI-1 than ET patients with no history of thrombosis (Bazzan et al. 1993). In septic patients, circulating PAI-1 can reach extraordinarily high levels (Colucci et al. 1985; Levi 2008; Semeraro et al. 2012) and lead to a strong and sustained suppression of fibrinolysis. In these patients, PAI-1 increase has been shown to be strongly associated with disseminated intravascular coagulation (DIC) and poor outcome (Pralong et al. 1989; Mesters et al. 1996; Lorente et al. 2014). Because platelets are extensively activated during sepsis and/or DIC, it might be surmised that platelet-derived PAI-1 contributes to fibrinolytic shutdown. It should be pointed out, however, that very high levels of PAI-1 can be detected in patients with severe sepsis, even in the absence of platelet consumption (Semeraro et al. 2015), suggesting that other cellular types are the principal source of circulating PAI-1 in this condition.

Taken together, the evidence strongly suggests that platelet PAI-1 is a major determinant of fibrinolytic resistance (Fig. 3) and probably plays a role in arterial thrombosis and in hemostasis, where it helps protect the plug from premature lysis.

Thrombin-Activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as plasma procarboxypeptidase B (proCPB) or procarboxypeptidase U (proCPU), is an important modulator of fibrinolysis (reviewed by Bajzar 2000; Foley et al. 2013). In the active form (TAFIa), it removes the C-terminal lysine residues from partially degraded fibrin, thereby reducing the binding of plasminogen to fibrin and thus efficiently inhibiting plasmin formation. TAFIa inhibits clot lysis with a threshold mechanism so that fibrinolysis is halted as long as TAFIa levels are at or above the threshold level (Leurs et al. 2004; Walker and Bajzar 2004). The threshold concentration of TAFIa, in turn, depends on the local fibrinolytic pressure; the higher the levels of plasminogen activator (s) (i.e., the higher the plasmin generation capacity), the higher the concentration of TAFIa needed to arrest clot lysis (Leurs et al. 2004; Walker and Bajzar 2004). At physiologically relevant t-PA concentrations, the amount of TAFIa capable of inhibiting the fibrinolytic process has been calculated to be less than 1 % of total TAFI (Walker and Bajzar 2004). The zymogen TAFI is activated through proteolytic cleavage by thrombin and plasmin. Thrombin is

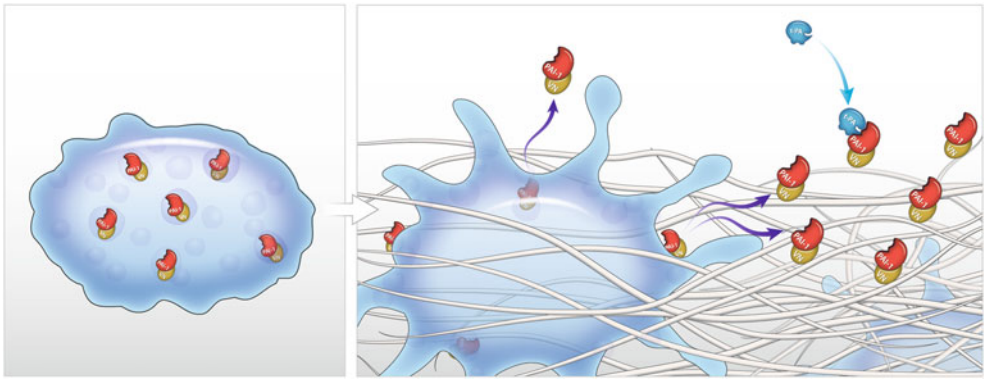

Fig. 3 The antifibrinolytic activity of platelet plasminogen activator inhibitor 1 (PAI-1). Resting platelets contain high amounts of PAI-1 stored in the α-granules in association with vitronectin (VN) (*left*). Upon activation, platelets release the PAI-1-vitronectin complex, which localizes and concentrates on the clot surface, through high-affinity vitronectin-mediated binding to fibrin, where it rapidly inhibits t-PA and prevents premature degradation of the clot (*right*)

a weak activator of TAFI but, when bound to thrombomodulin (TM), displays a 1000-fold higher catalytic efficiency (Bajzar et al. 1996a), which suggests that the thrombin–TM complex is the physiological activator of TAFI. It should be noted, however, that TM is localized on the endothelial surface, which means that at the site of vascular injury, within a nascent plug or thrombus, there is practically no TM apart from the small amount derived from circulating soluble TM (Ishii et al. 1990). Antifibrinolytic amounts of TAFIa can still be produced as a result of generation of huge amounts of thrombin within a forming clot (Mann 2003). Plasmin is a better activator of TAFI than thrombin, and its catalytic activity is further enhanced by binding to glycosaminoglycans (Foley et al. 2013). However, the role of plasmin as TAFI activator, within the frame of intravascular fibrinolysis regulation, is debated (Binette et al. 2007; Vercauteren et al. 2011; Semeraro et al. 2013).

Circulating TAFI derives mainly from the liver and its plasma concentration is approximately 10 μg/mL, with large interindividual variability (Gils et al. 2003). TAFI is also present in the α-granules of platelets, probably originating from endogenous synthesis in megakaryocytes (Mosnier et al. 2003). It is released on activation of platelets by thrombin and other agonists, and displays enzymatic characteristics similar to plasma TAFI. The concentration of platelet TAFI is low and accounts for only 0.1 % of plasma TAFI. However, at sites of platelet accumulation, the release of α-granule content from activated platelets might increase the local concentration of TAFI and enhance the antifibrinolytic effect. It has been calculated that the intraplatelet concentration of TAFI is comparable to that of plasma TAFI (Mosnier et al. 2003), and it is known that even a small increase in TAFI concentration can result in a reduction of clot lysis velocity (Bajzar et al. 1996b; Mosnier et al. 1998). Formal experimental evidence that platelet TAFI can add to plasma TAFI in the inhibition of clot lysis has recently been provided in an in vitro study (Schadinger et al. 2010).

In addition to TAFI release, platelets can potentiate TAFI-dependent inhibition of fibrinolysis through enhancement of thrombin-mediated TAFI activation (Fig. 4). Inside the hemostatic plug or a thrombus, particularly at arterial level, platelet-driven thrombin generation can reach levels that activate significant amounts of TAFI, even in the absence of TM. In an in vitro model of whole blood clot lysis, platelets enhanced thrombin-mediated TAFI activation in a concentration-dependent manner, leading to formation of highly resistant clots, independently of clot retraction (Carrieri et al. 2011). Interestingly, when blood was challenged with a low concentration of tissue factor, the TAFI-mediated antifibrinolytic effect of platelets was partly dependent on factor XI, but not on factor XII (Carrieri et al. 2011), lending further support to the purported role of thrombin-mediated factor XI activation in the generation of the "late" thrombin burst required for efficient TAFI activation (von dem Borne et al. 1995, 1997).

In several animal models of venous and arterial thrombosis, TAFI deficiency or the administration of TAFI inhibitors resulted in significant increase in thrombus lysis following treatment with fibrinolytic agents (reviewed by Morser et al. 2010; Colucci and Semeraro 2012), underscoring the role of TAFI activation in fibrinolytic resistance in vivo. Even though the role of platelets was not investigated in these studies, it is conceivable that, at least in arterial thrombi, activated platelets contribute to thrombus resistance through TAFI release and activation.

Factor XIII

Factor XIII (FXIII) is a pro-transglutaminase that, upon activation, introduces covalent ε(γ-glutamyl)lysyl bonds

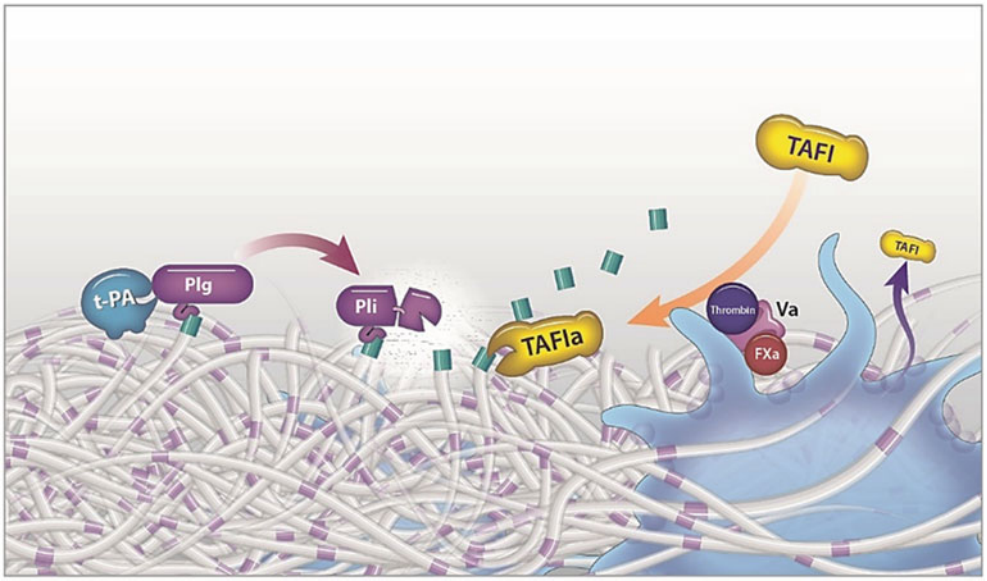

Fig. 4 Thrombin activatable fibrinolysis inhibitor (TAFI)-dependent antifibrinolytic effects of platelets. At the site of clot formation, stimulated platelets secrete TAFI from their α-granules, thereby increasing the local concentration of the inhibitor. Moreover, activated platelets promote the assembly of the prothrombinase complex through the exposure of procoagulant phospholipids and factor Va, thus enhancing thrombin generation. In turn, thrombin activates TAFI, which downregulates fibrinolysis by removing C-terminal lysine residues from fibrin and preventing the binding of plasminogen

between donor and acceptor polypeptides (reviewed by Muszbek et al. 2011). FXIII exists in two molecular forms that follow distinct activation routes and are localized in different compartments. Plasma FXIII (pFXIII) is a heterotetramer of two catalytic A and two carrier/inhibitory B subunits (FXIII-A_2B_2). Its activation requires both cleavage of the activation peptide by proteases (mainly thrombin) and calcium-dependent detachment of the B subunits. Cellular FXIII lacks the regulatory B component and consists of two A subunits only (FXIII-A_2), which assume an active configuration in the presence of increased levels of cytosolic calcium (Muszbek et al. 2011). Plasma FXIII is deemed extremely important in regulation of the fibrinolytic process. It stabilizes the fibrin clot and inhibits fibrinolysis by forming fibrin γ-chain dimers and α-chain polymers of high molecular weight, which are intrinsically more lysis resistant and support reduced binding of plasminogen. Moreover, crosslinking of plasma proteins, above all α_2-AP, but also TAFI and PAI-2, to fibrin further slows fibrin removal (Muszbek et al. 2011). Major sources of cellular FXIII are monocytes/macrophages and platelets, which contain about 60 ± 10 fg FXIII/cell (≈3 % of total platelet proteins) (Katona et al. 2001), making up to 50 % of the total blood FXIII activity (McDonagh et al. 1969). Platelet FXIII derives from megakaryocytes (Adany and Bardos 2003) and represents the only platelet transglutaminase activity (Mitchell et al. 2014). It is converted to the active form by raised calcium levels during platelet activation (Muszbek et al. 1995), although calpain- and platelet acid protease-dependent activation of cytosolic platelet FXIII have also been identified (Ando et al. 1987; Lynch and Pfueller 1988). Moreover, thrombin can accelerate activation of platelet FXIII in the extracellular environment (Muszbek et al. 1993). Most platelet FXIII is stored in the cytosolic fraction (Lopaciuk et al. 1976), and only minute amounts of FXIII endocytosed from plasma are contained in the α-granules (Marx et al. 1993), although pFXIII uptake has not been consistently described (McDonagh et al. 1969; Mitchell et al. 2014). It is not clear whether and how FXIII is released from platelets. It has been recently reported that ADP-activated and aggregated platelets can release FXIII from α-granules (Marx et al. 1993; Kreutz et al. 2015), but other works failed to identify FXIII in the platelet releasate (Joist and Niewiarowski 1973; Lopaciuk et al. 1976). It has been proposed that platelet FXIII is not released into the extracellular milieu, but is exposed on the surface of activated platelets (Kreager et al. 1988), with local distribution on the caps of PS-positive platelets and wider localization on PS-negative cells (Mitchell et al. 2014). However, no consensus exists because activation with thrombin-receptor activating peptide (TRAP-6) produced less than 5 % FXIII-A_2^+ platelets (Nagy et al. 2009) compared with more than 50 % positive platelets after activation with thrombin or dual agonists (TRAP-6/thrombin and collagen) (Mitchell et al. 2014). Probably the nature of the stimulus makes the difference, because dual stimulation with thrombin and collagen generates coated platelets, a subpopulation enriched in procoagulant proteins localized on the surface

through a platelet FXIII-dependent crosslinking process (Dale et al. 2002). Another potential mechanism for release of cytosolic FXIII is through microvesiculation of platelets activated with strong agonists (Holme et al. 1993).

Compared with pFXIII, the role of platelet FXIII in fibrinolysis regulation is more controversial. There is wide consensus that platelet FXIII-A_2 can catalyze crosslinking of fibrin chains and α_2-AP, as demonstrated in purified systems and in FXIII-deficient plasma (Reed et al. 1992; Hevessy et al. 1996), but its relative contribution in the presence of pFXIII is debated. Accelerated α_2-AP-fibrin crosslinking has been reported in platelet-rich plasma compared with platelet-poor plasma (Reed et al. 1992). Furthermore, extensive fibrin α-chain crosslinking has been found to correlate positively with platelet count, similar to the correlation with raised pFXIII concentration (Francis and Marder 1987). However, Hevessy et al. (1996) reported a negligible increase in crosslinking upon addition of normal platelets to normal plasma, raising concerns about the real contribution of platelet FXIII to fibrin stabilization. The difficulty in ascertaining the role of platelet FXIII is further amplified by the fact that platelets can influence FXIII activity independently of their own FXIII content. Indeed, activated platelets can provide a catalytic surface on which pFXIII crosslinking activity, especially on the α-chain and α_2-AP, is enhanced (Hevessy et al. 1996), which supports the antifibrinolytic activity of supplemental pFXIII (Dirkmann et al. 2012). Furthermore, stimulated platelets can bind activated pFXIII through plasmin-sensitive binding sites, probably active $\alpha_{IIb}\beta_3$ (Kreager et al. 1988; Cox and Devine 1994). It has been recently reported that the zymogen form of pFXIII can indirectly interact with the surface of activated platelets via the γ′-chain of $\alpha_{IIb}\beta_3$ receptor-bound fibrinogen (Nagy et al. 2009). In so doing, platelets can convey and concentrate both zymogen and active pFXIII to the site of clot formation. Recently, the role of platelet FXIII in fibrinolysis was studied under dynamic conditions (Mitchell et al. 2014), and platelets were found to increase the fibrinolytic resistance of FXIII-deficient plasma clots in a FXIII- and α_2-AP-dependent manner (Fig. 5). However, platelet FXIII contribution was no longer observed at pFXIII levels above 20 %. It is thus possible that platelet FXIII helps to stabilize the clots when pFXIII is lacking or diminished, as occurs in congenital deficiencies or during surgical and medical conditions associated with an acquired decrease in pFXIII levels (Tosetto et al. 1993). Data from humans congenitally deficient in FXIII, although scarce because of the rarity of the disease, seem to support such a theory because patients deficient in FXIII-B and characterized by low levels of pFXIII but normal cellular FXIII-A_2 experience milder bleeding symptoms than those with FXIII-A deficiency (Saito et al. 1990).

From a different perspective, platelet function, and particularly clot retraction, can be influenced by FXIII. Recently, it has been described that FXIII-A knockout mice exhibit greatly impaired retraction, which is not fully reversed by exogenous FXIII, indicating that it requires both extracellular and platelet FXIII activity (Kasahara et al. 2010). Indeed, extracellular proteins (fibrin and $\alpha_{IIb}\beta_3$) and intraplatelet cytoskeletal molecules (actin and myosin) are crosslinked by FXIII (Cohen et al. 1981), which is required for translocation of the retraction machinery to the sphingomyelin-rich rafts on the membrane of activated platelets (Kasahara et al. 2013). However, studies from FXIII-deficient patients have provided extremely conflicting results, as FXIII was found to inhibit, not affect, or even enhance clot retraction, probably because of differences between species and the use of diverse experimental conditions (Muszbek et al. 2011).

Polyphosphate

Polyphosphate (polyP) is a linear, negatively charged polymer of a few to several hundred inorganic orthophosphate residues and is abundantly distributed in the granules of human platelets (Ruiz et al. 2004) and mast cells (Moreno-Sanchez et al. 2012). Platelet-dense granules store millimolar concentrations of polyP, and lower amounts have also been detected in α-granules, colocalized with von Willebrand factor (Montilla et al. 2012). Platelet polyP is rather homogeneous and contains 60–100 phosphate residues (Müller et al. 2009). Stimulation with thrombin, ADP, or collagen results in platelet secretion of polyP in the extracellular milieu (Müller et al. 2009), reaching blood concentrations of 1–3 μM and, allegedly, even higher levels in platelet-rich thrombi (Ruiz et al. 2004). PolyP is deemed antifibrinolytic, based on multiple actions on both the coagulation and fibrinolytic systems. In plasma clot lysis assays, low micromolar concentrations of synthetic polyP delay t-PA- or u-PA-mediated fibrinolysis in a dose- and chain length-dependent manner. Maximum lysis time prolongation is achieved by polyP containing more than 45 units, representing the size of platelet polyP (Smith et al. 2006). The antifibrinolytic effect of polyP seems to be mediated by plasma TAFI because it is abolished by TAFIa inhibitors and disappears in TAFI-deficient plasma. Because polyP accelerates thrombin generation (Morrissey and Smith 2015), enhanced TAFI activation has been hypothesized. PolyP can also impair fibrinolysis by influencing the fibrin clot structure, although contrasting effects have been described. PolyP was found to either increase the turbidity of fibrin clots, by raising the fiber mass-to-length ratio (Smith and Morrissey 2008), or reduce it, giving rise to clots consisting of normal fibers organized

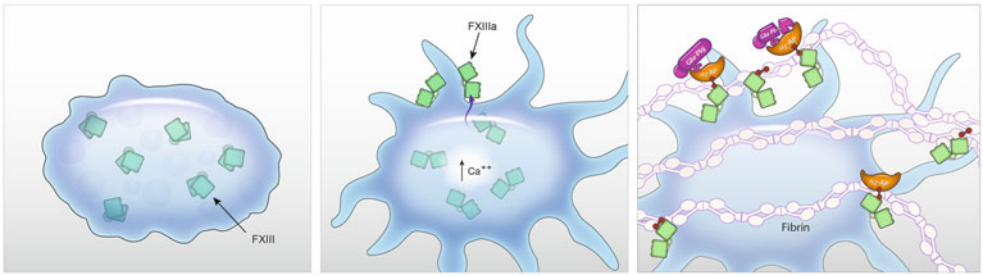

Fig. 5 Factor XIII (FXIII)-mediated antifibrinolytic activity of platelets. Resting platelets contain high amounts of inactive FXIII in their cytoplasm (*left*). Increased levels of cytosolic calcium consequent to platelet stimulation lead to conversion of FXIII into the active configuration (FXIIIa), which then translocates to the platelet surface (*middle*). Exposed FXIIIa forms fibrin γ-chain dimers and α-chain polymers, which are more lysis-resistant, and crosslinks α_2-antiplasmin (α_2-AP) to fibrin, which inhibits plasmin activity on the clot surface and competes with fibrin for plasminogen binding (*right*)

in dense knot-like regions and with ample pores (Mutch et al. 2010). In both cases, polyP downregulates fibrinolysis, probably through interfering with the binding of fibrinolytic proteins to fibrin. Recently, it was found that whole blood clots formed on thrombogenic surfaces under flow conditions are composed of thinner fibers, demonstrate increased clot retraction, and dissolve faster in the presence of the specific polyP inhibitor PPXbd, thus providing further evidence for and additional mechanistic insight into the antifibrinolytic activity of platelet polyP (Zhu et al. 2015).

No specific deficiency in polyP has been described in humans. However, very low polyP levels characterize several hereditary hemorrhagic diseases known as platelet dense granule storage pool deficiencies (e.g., Hermansky–Pudlak and Chediak–Higashi syndromes) (Hernández-Ruiz et al. 2009). In these human models of polyP deficiency, exogenous polyP can restore the platelet procoagulant potential (Müller et al. 2009), suggesting that defective polyP-mediated hemostatic mechanisms (e.g., protection of clots against fibrinolysis) could exacerbate the bleeding risk.

α_2-Antiplasmin

α_2-Antiplasmin (α_2-AP), also known as α_2-plasmin inhibitor, is the main physiological inhibitor of plasmin. It is a SERPIN of liver origin and circulates in blood at a concentration of 1 μM (reviewed by Rijken and Lijnen 2009). The inhibition of plasmin by α_2-AP is very rapid (second-order rate constant of 2×10^7–4×10^7 M^{-1} s^{-1}) and requires interaction of the inhibitor with both the active site and the high-affinity lysine binding sites of plasmin. During clot formation, α_2-AP is crosslinked to the fibrin α-chain by activated FXIII (Sakata and Aoki 1980). Several lines of evidence indicate that fibrin-bound α_2-AP plays a major role in regulating clot lysis (Sakata and Aoki 1982; Reed et al. 1990), unbound α_2-AP serving as inhibitor of plasmin generated in solution or released from the clot.

Platelet α_2-AP is stored in the α-granules (Plow and Collen 1981; Gogstad et al. 1983; Plow et al. 1989) and represents ≈0.05 % of the total inhibitor level in blood. To date there is no formal evidence that platelet α_2-AP makes any significant contribution to fibrinolytic resistance. Immunohistochemical studies, showing that the concentration of α_2-AP in human thrombi is approximately 25 % of that in plasma and that there is no difference between arterial and venous thrombi (Robbie et al. 1996), indicate that platelets add little to α_2-AP accumulation within thrombi.

Protease Nexin-1

Protease nexin-1 (PN-1), a member of the SERPIN superfamily, also known as serpin E2, is produced by many cell types and found in many organs. PN-1 inhibits a broad spectrum of serine proteases and has major pleiotropic functions. In vitro, PN-1 has been shown to inhibit several activated clotting factors as well as the fibrinolytic enzymes t-PA, u-PA, and plasmin. Heparin enhances the PN-1-mediated inhibition of many of its target proteases, including thrombin and FXIa, but has no significant effect on t-PA, u-PA, or plasmin inhibition by PN-1 (reviewed by Bouton et al. 2012).

PN-1 is barely detectable in plasma (Baker and Gronke 1986) but is present in platelets and monocytes (Mansilla et al. 2008). Most platelet PN-1 is stored in α-granules, only a small fraction being present on the surface of resting platelets (Boulaftali et al. 2010). In vitro, platelet-rich plasma clots display greater spontaneous as well as t-PA-induced lysis when formed in the presence of a neutralizing anti-PN-1 antibody or when prepared from PN-1-deficient mice (Boulaftali et al. 2011). Importantly, the antifibrinolytic effect of platelet PN-1 adds to that of PAI-1, because a more pronounced enhancement of lysis is seen upon neutralization of both inhibitors. Moreover, in a mouse model of $FeCl_3$-induced venular thrombosis, it was

reported that t-PA-induced thrombolysis was faster and led to a higher incidence of recanalization in PN-$1^{-/-}$ than in PN-$1^{+/+}$ mice (Boulaftali et al. 2011). An important feature of PN-1 is that it is able to inhibit fibrin-bound t-PA and fibrin-bound plasmin, suggesting that PN-1 plays a complementary role with PAI-1 and α_2-AP, which are much less efficient at inhibiting fibrin-bound enzymes.

Tissue Factor Pathway Inhibitor-2

Tissue factor pathway inhibitor-2 (TFPI-2), a homolog of TFPI-1, is a matrix-associated Kunitz-type serine proteinase inhibitor, previously designated as placental protein 5. TFPI-2 inhibits a broad spectrum of serine proteinases and is thought to play a role in the regulation of extracellular matrix digestion and remodeling (reviewed by Chand et al. 2005). With respect to the coagulation–fibrinolysis system, TFPI-2 has been shown to inhibit plasma kallikrein, FXIa, and plasmin (Petersen et al. 1996). In healthy subjects, the plasma levels of TFPI-2 are very low and unlikely to influence clotting or fibrinolysis. Platelets, by contrast, contain significant amounts of TFPI-2, probably derived from megakaryocytes (Vadivel et al. 2014). TFPI-2 is probably stored in the α-granules and released upon platelet stimulation. In an in vitro model of clot lysis, the release of TFPI-2 from activated platelets was shown to contribute significantly to fibrinolytic resistance, accounting for about 40 % of platelet-mediated clot lysis inhibition (Vadivel et al. 2014).

Zinc

Zinc is the second most abundant transition metal in blood and is considered an important mediator of hemostasis and thrombosis (reviewed by Vu et al. 2013). It binds numerous plasma proteins, thereby modulating their structure and function, and has been shown to influence platelet aggregation, coagulation, and fibrinolysis. Zinc has been reported to inhibit the enzymatic activity of t-PA and plasmin (Henderson et al. 2015) and to alter fibrin structure, giving rise to coarse clots composed of thicker fibers (Marx et al. 1987). The zinc concentration in plasma is 10–20 μM, but most of it is bound to albumin and other plasma proteins so that the free zinc concentration is 0.5–1 μM (Foote and Delves 1984). Platelets accumulate zinc in their cytoplasm and α-granules, such that the intracellular concentration of zinc is 30- to 60-fold higher than in plasma (Marx et al. 1993). Recently, it was reported that zinc ions delay the lysis of plasma clots, in a concentration-dependent manner, by decreasing t-PA-mediated plasminogen activation and plasmin-induced fibrin degradation (Henderson et al. 2015). On the basis of these findings, it can be hypothesized that the local surge in zinc levels at sites of platelet deposition and activation represents an additional mechanism of fibrinolytic resistance. To date, however, direct support for such a hypothesis is lacking.

Platelet-Derived Phospholipids

Platelet-derived phospholipids have been shown to inhibit fibrinolysis through different mechanisms. When incorporated into the clot, they form a diffusion barrier that decreases the penetration of t-PA and plasmin into the fibrin mesh; moreover, they compete with fibrin for the binding of fibrinolytic enzymes, thereby reducing plasminogen activation. In addition, phospholipids impair the protection of plasmin on the fibrin surface (Váradi et al. 2004) and make fibrin less susceptible to plasmin degradation (Günther et al. 1994). These antifibrinolytic effects can be largely reproduced with preparations of phospholipid vesicles consisting of phosphatidylserine and phosphatidylcholine (Váradi et al. 2004), which are the same phospholipids that promote tenase and prothrombinase assembly. These combined mechanisms result in marked inhibition of clot lysis, even in the presence of pharmacological concentrations of t-PA, and might play a role in thrombolysis resistance. In fact, strong activation or death leads to progressive loss of the platelet phospholipid content (Skarlatos et al. 1993), which could then accumulate within thrombi, as demonstrated by histochemical studies of human arterial thrombi (Váradi et al. 2004).

Platelets contain other factors capable of downregulating fibrinolysis, namely C1-inhibitor, which inhibits the plasminogen activators t-PA and u-PA (Huisman et al. 1995; Pannell et al. 2007), and histidine-rich glycoprotein, which acts as a competitive inhibitor of plasminogen (Lijnen et al. 1980). However, there is no evidence that the small platelet pool of these two proteins contributes to fibrinolytic resistance.

Platelet Profibrinolytic Properties

Plasminogen and Its Receptor(s)

Platelets have long been known to contain and/or express several profibrinolytic proteins, namely u-PA, t-PA, and plasminogen (Jeanneau and Sultan 1988; Brisson-Jeanneau et al. 1990; Wang et al. 1994; Coppinger et al. 2004; Maynard et al. 2007; Veljkovic et al. 2009). Plasminogen, in particular, is contained in platelet α-granules (Maynard et al. 2007) and is released upon thrombin stimulation (Coppinger et al. 2004). One of the most important functions

of platelets in mediating fibrinolysis is to provide a surface on which localized fibrinolytic activity can be generated. Platelets, like many other cell types, express specific receptors that bind plasminogen and enhance its activation (Miles and Plow 1985; Adelman et al. 1988; Loscalzo et al. 1995). The plasminogen-binding capacity of platelets is high and increases severalfold following stimulation by thrombin (Miles and Plow 1985). The interaction of plasminogen with platelets is mediated by the lysine binding sites located in the kringle domains of plasminogen and the C-terminal lysine residues on the platelet surface, and can be inhibited by the lysine analog EACA and by carboxypeptidase B (Miles and Plow 1985; Miles et al. 1988; Adelman et al. 1988; Horne III et al. 2005). The exact nature of the protein(s) exposing C-terminal lysines on the platelet surface has not been conclusively established. Available studies indicate that plasminogen interaction with platelets involves the integrin $\alpha_{IIb}\beta_3$ and point to surface-bound fibrin(ogen) (via $\alpha_{IIb}\beta_3$) as the main plasminogen binding site (Miles and Plow 1985; Miles et al. 1986; Adelman et al. 1988). In thrombin-stimulated platelets, fibrin associated with the platelet surface is probably the prominent site of plasminogen binding through the increased exposure of C-terminal lysines (Miles and Plow 1985; Miles et al. 1986), although the possibility that thrombin exposes C-terminal lysines in a protein other than fibrin cannot be excluded (Horne III et al. 2005). In the case of platelet agonists other than thrombin, which have been shown to increase plasminogen interaction with platelets by some investigators (Adelman et al. 1988) but not by others (Miles and Plow 1985; Miles et al. 1986), $\alpha_{IIb}\beta_3$-bound fibrinogen appears to play a major role. Concerning unstimulated platelets, it is possible that plasminogen interacts directly with $\alpha_{IIb}\beta_3$, as originally reported by Miles et al. (1986). Whether platelet $\alpha_{IIb}\beta_3$ undergoes proteolysis or some other change to reveal C-terminal lysine residues remains unclear (Miles and Parmer 2013). Whatever the binding mechanism, when in association with the platelet surface, plasminogen assumes an open conformation that is more readily cleaved to plasmin by plasminogen activators (Miles and Parmer 2013). Moreover, cell-associated plasmin is considerably protected from inhibition by α_2-AP (Hall et al. 1991), suggesting that platelets provide an efficient profibrinolytic surface. The possible relevance of these mechanisms is suggested by recent findings in models that better mimic physiological conditions. Whyte et al. (2015), using an in vitro model of thrombus formation under physiological flow rates, showed that plasminogen accumulates within the platelet-rich thrombus and supports t-PA- or u-PA-induced thrombolysis in a flow-dependent fashion. Plasminogen binds to platelets mainly in a fibrin (ogen)-dependent manner, particularly on PS-expressing platelets, where it localizes on protruding caps displaying greater plasmin generation capacity. These in vitro findings were largely confirmed in vivo in a mice model of laser-induced mesenteric vein thrombosis (Brzoska et al. 2015). In fact, the authors found that plasminogen accumulates in a time-dependent manner in the center of the microthrombus, which coincides with the localization of strongly activated PS-exposing platelets and fibrin. Plasminogen accumulation is dependent on lysine binding sites and requires plasmin formation. Interestingly, both in vitro and in vivo studies provide evidence suggesting that spontaneous plasminogen activation takes place within the thrombus, even though it does not result in detectable thrombolysis during the observation period.

Plasminogen Activators and Their Receptors

Both u-PA and t-PA are involved in platelet-mediated fibrinolysis. Platelets, besides containing t-PA (Jeanneau and Sultan 1988; Brisson-Jeanneau et al. 1990; Wang et al. 1994), have been shown to express specific binding sites for t-PA (Vaughan et al. 1989) and, importantly, platelet-bound t-PA manifests enhanced catalytic activity (Deguchi et al. 1985; Ouimet et al. 1994; Loscalzo et al. 1995). Although neither the nature of the platelet receptor nor the structure(s) in the t-PA molecule that are responsible for binding to platelets have been identified, the co-localization of plasminogen and its activator on the platelet surface boosts plasmin formation and thereby promotes pericellular fibrinolysis.

It has long been known that u-PA is associated with normal platelets and their external membranes; it is estimated that about 20 % of the u-PA intrinsic to blood is tightly associated with platelets. Platelet uptake of scu-PA from the ambient plasma has also been demonstrated (Park et al. 1989; Gurewich et al. 1993, 1995). Platelets are able to bind scu-PA through a still-unidentified surface receptor that is different from the classical u-PA receptor (u-PAR) expressed by other cells (Vaughan et al. 1990; Jiang et al. 1996). Platelet-bound scu-PA can be activated by pre(kallikrein) co-localized on the cell surface (Gurewich et al. 1993; Loza et al. 1994) or by platelet-bound plasmin (ogen) (Baeten et al. 2010). The latter mechanism provides a plausible explanation for the interesting observation that platelets enhance clot lysis by scu-PA, but not by u-PA or a mutated scu-PA that cannot be activated to u-PA (Baeten et al. 2010). In thrombin-activated platelets, a complex mechanism has been described whereby thrombin inactivates scu-PA, which is then reactivated by a platelet protease, suggested to be cathepsin C (Nauland and Rijken 1994; Lenich et al. 1997).

Platelet profibrinolytic activity mediated by u-PA is believed to play a major role in a rare bleeding disorder named Quebec platelet disorder (QPD) (reviewed by

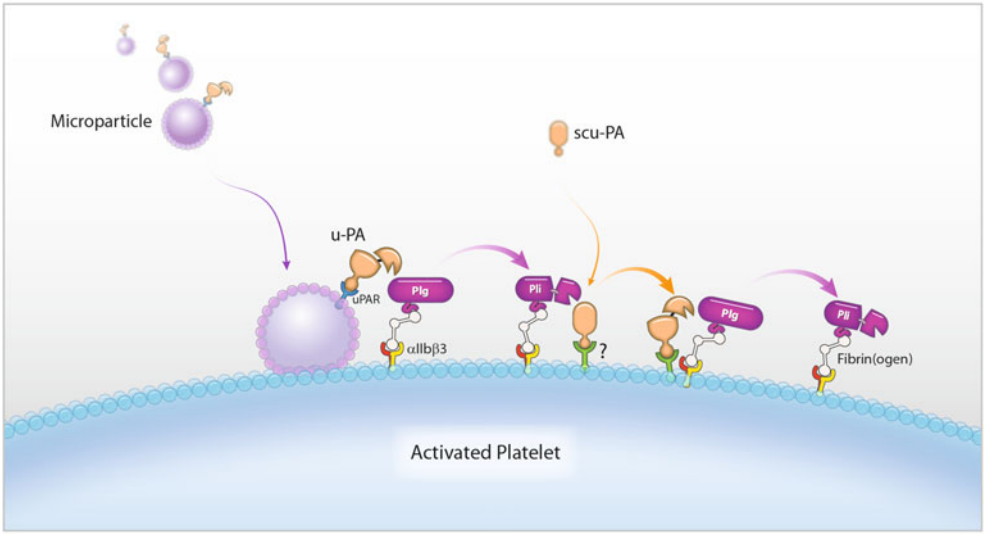

Fig. 6 Main profibrinolytic properties of platelets. Platelets provide a suitable surface for the localization, assembly, and activation of fibrinolytic enzymes. Indeed, plasminogen binds to the platelet surface through the $\alpha_{IIb}\beta_3$-fibrin(ogen) complex and assumes a conformation that is more readily converted to plasmin by plasminogen activators. u-PA expressed on circulating microparticles can activate plasminogen on the platelet surface. Plasmin, in turn, generates additional u-PA activity by cleaving scu-PA, which is bound to platelets by not yet identified receptors. Plasminogen can also be activated by surface-bound t-PA (not shown)

Blavignac et al. 2011). QPD is an autosomal dominant platelet disease with unusual clinical features; it is associated with moderate to severe delayed bleeding, which can be controlled with fibrinolytic inhibitors such as tranexamic acid. The hallmark feature of this disease is the exceedingly high content of u-PA in megakaryocytes and platelets, combined with normal levels of plasma and urinary u-PA. The u-PA content in QPD platelets has been estimated to be more than 100-fold higher than in normal platelets. The enzyme is present in the free form and in complex with PAI-1, suggesting that QPD platelets co-store u-PA with PAI-1 in α-granules (Kahr et al. 2001). The presence of active u-PA is thought to arise from the co-localization of scu-PA and plasminogen within α-granules, which leads to plasmin generation, as indicated by the presence of plasmin–antiplasmin complexes in QPD platelets (Sheth et al. 2003). Intracellular plasmin, in turn, appears to be responsible for the degradation of α-granule storage proteins, including factor V and fibrinogen. Although the bleeding diathesis of QPD patients involves several pathogenetic mechanisms, it is conceivable that the local release of active u-PA into the clot leads to premature lysis of the hemostatic plug (Diamandis et al. 2006; Blavignac et al. 2011).

Other Profibrinolytic Properties

There are other reported mechanisms whereby platelets exert profibrinolytic effects. Dejouvencel et al. (2010), showed that platelet-bound plasminogen is converted to plasmin by u-PA expressed on microparticles derived from monocytes or endothelial cells. This u-PA-driven cross-talk mechanism generates plasmin in situ with higher efficiency than plasminogen activation by u-PA expressed on the same cell surface, thus highlighting its potential relevance in fibrinolysis and matrix proteolysis induced by inflammatory cells or cell-derived microparticles. Moreover, platelets have been shown to upregulate u-PA synthesis by endothelial cells in vitro, an effect that has been attributed to platelet-bound u-PA following its interaction with u-PAR on endothelial cells (Camoin-Jau et al. 2002). Furthermore, platelets can hasten fibrinolysis by indirect mechanisms through the release of platelet factor 4 (PF4) and, paradoxically, polyP. Indeed, as shown by Mosnier (2011), PF4 modulates the substrate specificity of the thrombin–TM complex by inhibiting TAFI activation but not protein C activation, thereby preventing generation of the antifibrinolytic activity of TAFIa. PolyP may drive fibrinolysis by providing a surface for activation of the contact system (Müller et al. 2009). A schematic representation of the main profibrinolytic properties of platelets is shown in Fig. 6.

Effects of Fibrinolysis on Platelets

Plasmin has been extensively investigated in vitro and shown to exert complex and paradoxical effects on platelets, being able to both activate and inhibit platelet functions (reviewed by Coller 1990; Pasche and Loscalzo 1991).

Several variables influence the platelet response to plasmin, including plasmin concentration, temperature, exposure time, and platelet environment. According to numerous reports, high concentrations of plasmin trigger platelet aggregation, calcium mobilization, protein phosphorylation, and activation of protein kinase C and phospholipase C (Schafer et al. 1986; Coller 1990; Pasche and Loscalzo 1991; Loscalzo et al. 1995; Rabhi-Sabile and Pidard 1995; Rabhi-Sabile et al. 1998). As to the mechanism(s) of platelet stimulation, plasmin was shown to activate the protease-activated receptor 4 (PAR-4), but not PAR-1 (Quinton et al. 2004), as previously suggested (Kuliopulos et al. 1999). Moreover, plasmin-induced ADP release might further enhance platelet aggregation by high-dose plasmin (Ishii-Watabe et al. 2000).

In contrast with these findings, exposure to low plasmin concentrations was found to decrease platelet activation in response to several agonists (Coller 1990; Loscalzo et al. 1995; Kinlough-Rathbone et al. 1997). Some investigators have related this effect to plasmin-induced stimulation of adenylate cyclase activity (Adnot et al. 1987) and others to impairment of arachidonic acid metabolism (Schafer and Adelman 1985). Moreover, plasmin was shown to synergize with prostaglandin I_2 (PGI_2) in inhibiting thrombin- and ADP-induced platelet activation (Schafer et al. 1987), a finding of potential importance for the control of platelet accrual at sites of vascular injury, where the release of t-PA and PGI_2 by adjacent endothelial cells is generally increased. Considering the broad specificity of plasmin, it is not surprising that plasmin-induced proteolysis of platelet receptors such as $\alpha_{IIb}\beta_3$ and GPIb has long been considered a major mechanism of platelet dysfunction. The degradation of platelet surface glycoproteins has been documented with both washed platelets (Schafer and Adelman 1985; Coller 1990; Winters et al. 1990; Pasche and Loscalzo 1991; Pasche et al. 1994; Loscalzo et al. 1995) and platelet-rich plasma and is associated with impaired responses to specific platelet agonists (Adelman et al. 1985; Pasche et al. 1994). In addition to these mechanisms, inactivation of the PAR-1 thrombin receptor by plasmin (Kimura et al. 1996) and the generation of fibrin(ogen) degradation products (Torr et al. 1990; Hoffmann and Janssen 1992; Gouin et al. 1992; Huang et al. 1998) have also been suggested to play a role in platelet dysfunction.

Other important factors influencing the platelet response to plasmin in vitro are temperature and exposure time. Lowering the temperature to 22 °C dramatically increases the sensitivity of platelets to plasmin so that low plasmin concentrations result in platelet activation, and very low plasmin doses potentiate the platelet response to other agonists (Lu et al. 1991). Likewise, prolonged (>60 min) exposure of platelets to low doses of plasmin leads to platelet aggregation and enhanced expression of procoagulant activity (Ervin and Peerschke 2001). The influence of temperature on the platelet response to plasmin could be of clinical relevance during open-heart cardiopulmonary bypass surgery (CPB), when blood temperature is reduced. Patients undergoing CPB may experience marked blood loss and prolonged bleeding, which has been attributed to complex hemostatic defects including heightened fibrinolytic activity and platelet activation/consumption (Tanaka et al. 1987; Paparella et al. 2004). It is plausible, therefore, that temperature-dependent, plasmin-induced platelet activation contributes to thrombocytopenia and blood loss during CPB. Consistent with this hypothesis, antifibrinolytic agents have been shown to reduce blood loss in patients undergoing CPB (Koster et al. 2015).

Modulation of platelet function by plasmin could be particularly relevant during thrombolytic therapy. Theoretically, both plasmin-induced platelet activation and platelet dysfunction might occur in vivo in patients undergoing therapeutic thrombolysis. Fibrinolysis-induced platelet activation could represent a potential mechanism for late reperfusion or early reocclusion after initial successful thrombolysis (Leopold and Loscalzo 1995), as also suggested by the observation that the offending thrombus in reoccluded vessels is composed primarily of platelets with very little fibrin (Eccleston and Topel 1995). On the other hand, plasmin-induced platelet dysfunction might contribute to the hemorrhagic complications known to be associated with thrombolytic therapy. Literature data demonstrate that the plasma concentration of platelet-derived substances is often elevated after thrombolysis (Coller 1990; Pasche and Loscalzo 1991; Rasminis et al. 1992; Salvoni et al. 1994; Frandsen et al. 1996). However, there is no consensus about the dynamics of ex vivo platelet status during thrombolytic treatment, with evidence of both platelet activation and inhibition (Coller 1990; Bertolino et al. 1992; Karlberg et al. 1993; Bihour et al. 1995; Lu et al. 1995). Such discrepancies could be related to several factors such as the type of thrombolytic agent, the techniques used to assess platelet function, and the timing of blood sampling. Clinical trials comparing different thrombolytic agents at specified times after thrombolysis have provided a better picture of platelet changes. Early after starting thrombolysis (1–3 h), a reduction in agonist-induced platelet aggregation was found (Moser et al. 1999), the effect being stronger with streptokinase and reteplase (a mutant of t-PA with prolonged half-life) than with alteplase. Analysis of platelet surface receptors (3–6 h) revealed a decrease in $\alpha_{IIb}\beta_3$, P-selectin, and platelet endothelial cell adhesion molecule-1 (PECAM-1), probably reflecting plasmin-induced cleavage (Gurbel et al. 1998; Moser et al. 1999). In contrast, at later intervals (>12 h with peak at 24 h), a marked increase in specific receptor expression was found, which was most pronounced for PECAM-1, very late antigen-2 (integrin $\alpha_2\beta_1$, GPIa/IIa),

and $\alpha_{IIb}\beta_3$. Again, this effect was stronger with reteplase than with alteplase and was associated with an increased aggregation response (Gurbel et al. 1998). A similar effect was reported by Bihour et al. (1995) within the first 72 h after treatment with reteplase, albeit in a small group of patients. Serebruany et al. (2003) compared the effect on platelets of alteplase and tenecteplase (a triple-point mutant of t-PA with longer half-life, enhanced fibrin specificity, and resistance to PAI-1). In vitro, both drugs caused significant inhibition of conventional and whole blood aggregation and decreased the expression of $\alpha_{IIb}\beta_3$, PECAM-1, vitronectin receptor, and the formation of platelet–monocyte aggregates, with tenecteplase exhibiting a stronger antiplatelet activity than alteplase. In patients undergoing thrombolytic therapy, a significant decrease in plasma levels of platelet-released biomarkers was observed in the first 3 h after thrombolysis, which was more marked with tenecteplase than with alteplase. It was suggested that tenecteplase exhibits significantly more powerful antiplatelet properties than alteplase (Serebruany et al. 2003), although ex vivo data on the expression of platelet receptors are lacking. Unfortunately, longer time intervals after thrombolysis (12–24 h) were not investigated in this study and thus it remains unknown whether tenecteplase has the same platelet activating properties as reteplase. The precise mechanisms underlying the platelet changes caused by thrombolytic agents in vivo as well as their impact on clinical manifestations associated with thrombolysis (bleeding, late reperfusion, early reocclusion) remain uncertain. Nevertheless, whatever the mechanism, understanding the temporal relation of phasic changes of platelet function to the administration of fibrinolytic therapy still represents an important task, particularly when considering the concomitant use of antiplatelet drugs to enhance reperfusion.

Putting All Together

It is widely acknowledged that platelets make clots resistant to fibrinolysis. In fact, both in vitro and in vivo studies indicate that platelet-rich thrombi are more resistant than platelet-poor thrombi to both pharmacological thrombolysis and endogenous fibrinolysis. This body of evidence could lead to the conclusion that the antifibrinolytic activities of platelets prevail over the profibrinolytic ones. However, any assumption about the role of platelets based on a mere balance between pro- and antifibrinolytic properties might be flawed. Thanks to recent developments in intravital microscopy imaging approaches, we have learned that platelet activation and function within a thrombus in vivo is heterogeneous both in space and time (Ivanciu and Stalker 2015) and that different platelet populations could play different roles at different times. In order to be effective, the hemostatic plug must form as quickly as possible and must survive long enough to permit the healing process. This implies that the fibrinolytic process must be kept in check for quite a long period to avoid untimely removal of the hemostatic plug, and be activated at the proper time. Platelets are likely to participate in the regulation of fibrin removal by virtue of their multiple pro- and antifibrinolytic properties. Based on current in vitro and in vivo evidence, and on recent findings on the spatiotemporal regulation of platelet and coagulation activation (Ivanciu and Stalker 2015), one can envision the following scenario for how platelets regulate fibrinolysis. Following vascular damage, platelets and fibrin accumulate at the site of injury rapidly and simultaneously (Falati et al. 2002). As the aggregate grows, platelets become activated in a graded fashion extending from the injury site, probably because gradients of platelet agonists are established within the evolving platelet mass. In the in vivo formed thrombus, two main regions can be identified on the basis of platelet activation state, platelet packing density, porosity, thrombin activity, and fibrin deposition: the inner core and the outer shell. The inner core is localized adjacent to the injury site and consists of fully activated, densely packed platelets. High thrombin activity and fibrin deposition characterize this area. Here, fibrinolytic resistance is largely dependent on clot retraction, release of PAI-1 and other antifibrinolytic factors, and enhancement of thrombin formation, with the consequent activation of TAFI and FXIIIa-mediated crosslinking of α_2-AP to fibrin. In addition, dense platelet packing creates sheltered microenvironments between platelets, which, on the one hand, are important harbors for platelet releasates, and, on the other hand, greatly reduce the transport of plasma solutes into the spaces between platelets. The outer shell surrounds the inner core and consists of loosely packed, partially activated platelets, which retain their α-granules, generate little thrombin activity and promote little, if any, fibrin formation. Conceivably, this region displays low fibrinolytic resistance and is susceptible to disaggregation by shear stress forces. A third distinct population of activated platelets is found close to collagen fibers and as patches around the thrombus (Heemskerk et al. 2013). These platelets are characterized by surface-exposed PS, rounded structure, raised cytosolic Ca^{2+}, and the ability to bind coagulation factors (Munnix et al. 2007; Berny et al. 2010). They support thrombin generation and fibrin formation but, at variance with fully activated platelets within the inner core, are unlikely to induce clot retraction because they do not exhibit activated $\alpha_{IIb}\beta_3$ (Kulkarni and Jackson 2004; Munnix et al. 2009). Thus, in these specific areas, fibrinolytic resistance is mainly dependent on thrombin formation and release of intraplatelet inhibitors.

In physiological hemostasis, the mechanisms leading to fibrinolytic resistance are limited in time because thrombin generation and the related antifibrinolytic effects gradually

decay as a result of the action of natural inhibitors. Moreover, the fibrinolytic inhibitors accumulated within the clot, chiefly PAI-1 and crosslinked α_2-AP, become progressively saturated by the continuous influx of plasminogen activators released by endothelial cells or delivered by circulating microparticles (Dejouvencel et al. 2010; Lacroix et al. 2012) and by the generation of plasmin. This leads to a switch from an anti- to a profibrinolytic state and the true lytic phase may eventually take place. Platelets might also participate in this lytic phase, allegedly through plasminogen binding and enhancement of plasmin generation. As shown by in vivo studies, plasminogen binding commences early after vessel injury, is highest within the platelet-rich inner core of the nascent thrombus, and is associated with plasmin formation (Brzoska et al. 2015). However, plasmin generation does not lead to detectable thrombus lysis in the early phases (Brzoska et al. 2015), supporting the idea that at this stage there is an excess of inhibitors within the thrombus. With time, the continuous binding and activation of plasminogen can overcome the inhibitors and initiate the lytic phase. In addition, as already pointed out, platelets can further modulate fibrinolysis through cross-talk with other blood and vascular cells and cell-derived microparticles. It is noteworthy that, in a model of arterial thrombosis, fibrin degradation coincides with leukocyte binding to the thrombi via interaction of leukocyte P-selectin glycoprotein ligand-1 with P-selectin on the surface of activated platelets (Bai et al. 2009). Better knowledge of the complex spatiotemporal regulation of endogenous fibrinolysis and pharmacological thrombolysis by platelets has important pathophysiological implications and should help improve therapeutic strategies for disease states encompassing bleeding and thrombotic disorders.

Take Home Messages

1. Platelets have the potential to both down- and upregulate the fibrinolytic process
2. Antifibrinolytic effects of platelets are mediated mainly by:
   a. Clot retraction, which makes the fibrin mesh stiffer and more resistant to lysis
   b. Release of several inhibitors of fibrinolysis, including huge amounts of plasminogen activator inhibitor-1 (PAI-1)
   c. Enhancement of thrombin formation, which in turn modifies the fibrin structure to make the clot denser and more resistant to fibrinolysis. Thrombin, moreover, activates two important enzyme precursors, namely factor XIII and thrombin-activatable fibrinolysis inhibitor (TAFI), which stabilize the clot and inhibit plasmin generation on the fibrin surface
3. Platelet profibrinolytic activities include:
   a. Release of plasminogen activators
   b. Binding of plasminogen and enhancement of plasminogen activation
4. In the hemostatic process, the antifibrinolytic activities of platelets contribute to protection of the hemostatic plug from premature lysis. At later stages, platelets might hasten fibrinolysis through enhancement of plasminogen activation
5. In thrombotic diseases, particularly arterial thrombosis, platelets increase resistance to fibrinolysis/thrombolysis, thus favoring thrombus persistence

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Platelets and Immunity

Ingrid Slaba and Paul Kubes

Abstract

Over the last several decades, a new paradigm of platelet function has evolved. Platelets, long forgotten hemostatic cells, were demonstrated to be versatile immune effector cells engaged in every compartment of the immune system. Platelets express multiple immune receptors, such as toll-like receptors, allowing them to sense both pathogen- and danger-associated signals. Upon activation, platelets release a large array of biologically active molecules, like cytokines, chemokines, and growth factors, many of which are delivered in platelet-derived microparticles. Rich platelet transcriptome allows for signal-dependent translation and protein synthesis, and platelets are considered the main source of circulating microRNA. Platelets avidly interact with immune cells, endothelial cells, neutrophils, and monocytes in particular. Platelets were shown to propagate and modulate the inflammatory response of other immune cells in sterile inflammatory diseases, such as atherosclerosis and metabolic diseases, acute lung injury, ischemia reperfusion, and autoimmune diseases. Platelets are directed to the sites of infection and directly interact with pathogens. They contribute to elimination of pathogens by phagocytosis, release of microbicidal peptides, and signaling to other immune cells. Their interaction with neutrophils triggers release of neutrophil extracellular traps, a potent mechanism to entrap bacteria in flowing blood. Platelets were recently shown to be involved in adaptive immunity and possibly link innate and adaptive responses together. Fast flowing in the bloodstream, readily available for cellular interactions, able to sense signals from pathogens and damaged tissue, and packed with multiple immune mediators, platelets arise as immune sentinels forming an integral part of our immune system.

List of Symbols, Abbreviations, and Nomenclature

ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ALI	Acute lung injury
ATP	Adenosine triphosphate
C1	C1 esterase inhibitor
CCL2	Chemokine (C–C motif) ligand 2, or monocyte chemotactic protein 1 (MCP-1)
CCL3	Chemokine (C-C motif) ligand 3, or macrophage inhibitory protein 1 alpha (MIP-1α)

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CCL5	Chemokine (C-C motif) ligand 5, or regulated on activation, normal T cell expressed and secreted (RANTES)
CCL7	Chemokine (C-C motif) ligand 7, or monocyte chemotactic protein 3 (MCP-3)
CCL17	Chemokine (C-C motif) ligand 17, or thymus- and activation-regulated chemokine (TARC)
CCR1	C–C chemokine receptor type 1, or CD191
CCR3	C–C chemokine receptor type 3, or CD193
CCR4	C–C chemokine receptor type 4, or CD194
CD11a/CD18	Lymphocyte function-associated antigen 1, or LFA-1
CD11b/CD18	Macrophage antigen 1, or Mac-1
CD14	Cluster of differentiation 14
CD154	Cluster of differentiation 154, or CD40L
CD31	Cluster of differentiation 31, or PECAM-1
CD36	Cluster of differentiation 36
CD40	Cluster of differentiation 40
CD40L	Cluster of differentiation 40 ligand; or CD154
CD41	Cluster of differentiation 41
CD8+	T cells expressing cluster of differentiation 8 on their surface
circRNA	Circular ribonucleic acid
CLEC-2	C-type lectin-like receptor 2
CXCL1	Chemokine (C-X-C motif) ligand 1, or GRO1 oncogene, GROα, KC, neutrophil-activating protein 3 (NAP-3), melanoma growth stimulating activity, alpha (MGSA-α)
CXCL4	Chemokine (C-X-C motif) ligand 4, or platelet factor 4 (PF4)
CXCL5	Chemokine (C-X-C motif) ligand 5, or ENA-78
CXCL7	Chemokine (C-X-C motif) ligand 7, or beta thromboglobulin 1 (βTG1), pro-platelet basic protein (PPBP)
CXCL8	Chemokine (C-X-C motif) ligand 8, interleukin 8 (IL-8)
CXCL16	Chemokine (C-X-C motif) ligand 16
CXCR4	C-X-C chemokine receptor type 4, or CD184
DAMPs	Damage-associated molecular patterns
DC(s)	Dendritic cell(s)
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalitis
EMCV	Encephalomyocarditis virus
ENA-78	Epithelial-derived neutrophil-activating peptide 78, or CXCL5
Factor V, VII, XI, XIII	Coagulation factors V, VII, XI, XIII
FcγRIIA	Immunoglobulin receptor Fc gamma IIA, or CD32
FoxP3	Forkhead box P3, or scurfin
FPR	Formyl peptide receptor
FPR1	*N*-formyl peptide receptor 1
FPR-L1	*N*-formyl peptide receptor like 1
GPIb	Glycoprotein Ib
GPIb-V-IX	Membrane glycoprotein Ib, V and IX complex
GPIbα	Glycoprotein Ib alpha
GPIIb	Glycoprotein IIb, or integrin αIIb
GPIIbIIIa	Glycoprotein IIbIIIa
GPVI	Glycoprotein VI
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HMGB-1	High-mobility group box 1
HSPs	Heat shock proteins
ICAM-1	Intercellular adhesion molecule 1
ICAM-2	Intercellular adhesion molecule 2, or CD102
IFNα	Interferon alpha
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukin-1 beta
IL-1R	Interleukin-1 receptor
IL-1α	Interleukin-1 alpha
IL-6	Interleukin 6
IL-8	Interleukin 8, or CXCL8
IL-10	Interleukin 10
IL-12p70	Interleukin 12, or cytotoxic lymphocyte maturation factor (CLMF)
JAM-3	Junctional adhesion molecule 3
JAM-C	Junctional adhesion molecule C
LFA-1	Lymphocyte function-associated antigen 1; or CD11a/CD18
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
Mac-1	Macrophage antigen 1, or CD11b/CD18
MD2	Lymphocyte antigen 96
MCP-1	Monocyte chemotactic protein 1, or chemokine (C-C motif) ligand 2 (CCL2)
MCP-3	Monocyte chemotactic protein 3, or chemokine (C-C motif) ligand 7 (CCL7)
MHC	Major histocompatibility complex
MIP-1α	Macrophage inhibitory protein 1 alpha, or chemokine (C-C motif) ligand 3 (CCL3)

mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MS	Multiple sclerosis
MyD88	Myeloid differentiation primary response gene 88
NET(s)	Neutrophil extracellular trap(s)
NFkB	Nuclear factor kappa B
NK cells	Natural killer cells
OLT	Orthotopic liver transplantation
P2Y12	Platelet adenosine diphosphate (ADP) receptor 12
PAF	Platelet-activating factor
PAMPs	Pathogen-associated molecular patterns
PAI-1	Plasminogen activator inhibitor 1
PDGF	Platelet-derived growth factor
PECAM-1	Platelet-endothelial cell adhesion molecule 1
PF4	Platelet factor 4, or CXCL4
PPARγ	Peroxisome proliferator-activated receptor gamma
pre-mRNA	Pre-messenger ribonucleic acid
PRRs	Pathogen recognition receptors
PSGL-1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
RANTES	Regulated on activation, normal T cell expressed and secreted, or CCL5
RES	Reticuloendothelial system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SSRI	Selective serotonin reuptake inhibitors
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor beta
TLR1	Toll-like receptor 1
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR9	Toll-like receptor 9
TLR(s)	Toll-like receptor(s)
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
tRNA	Transfer ribonucleic acid
T_{reg}	T regulatory cells
TSP-1	Thrombospondin-1
TXA_2	Thromboxane A2
VASP	Vasodilator-stimulated phosphoprotein
VCAM-1	Vascular cell adhesion molecule 1; or CD106
VEGF	Vascular endothelial growth factor
vWf	Von Willebrand factor
α2β1	Integrin α2β1, or GPIa/IIa
α5β1	Integrin α5β1
α6β1	Integrin α6β1
αIIβ3	Intergrin αIIβ3, or glycoprotein IIb/IIIa, GPIIbIIIa
αvβ3	Integrin αvβ3, or CD51/CD61
βTG	Beta thromboglobulin, or CXCL7

Introduction

The role of platelets as principal cells of thrombosis and hemostasis has been studied for several decades and is quite well understood. However, there is increasing evidence that platelets are active players in many other physiological functions, including inflammation, infection, wound healing, maintenance of endothelial barrier function, angiogenesis, and tumor metastasis (Gay and Felding-Habermann 2011; Nachman and Rafii 2008; Nurden 2011; Sabrkhany et al. 2011; Semple et al. 2011; Smyth et al. 2009; Yeaman 2010).

The role of platelets in immunity, infection, and inflammation is now being studied intensively, and results of this research are substantially changing our understanding of platelet functions. The ability of the platelet to adhere, form aggregates, and release mediators creates the perfect opportunity for platelets to be actively involved in the inflammatory response. Also, platelets are abundant in the bloodstream and readily available for activation and adhesive cellular interactions. Platelets were shown to become activated by various inflammatory mediators, be the source of chemokines and cytokines, express adhesion molecules, recruit immune cells to sites of vascular injury and inflammation, and directly kill bacteria and parasite-infected cells (Andonegui et al. 2005; Bombeli et al. 1998; Faull et al. 1994; McMorran et al. 2012; Semple et al. 2011; Smyth et al. 2009; Yeaman 2010; Zarbock et al. 2007). Platelets binding to adherent and activated neutrophils induce neutrophil extracellular trap (NET) formation to ensnare bacteria in septic blood. NETs consist of chromatin, histones, and elastase, and besides their powerful protection against pathogens, they also might cause injury to the host (Clark et al. 2007; McDonald et al. 2012). Interestingly, NETs were documented also in sterile conditions, like thrombosis (Fuchs et al. 2010, 2012). It is becoming evident that platelets are not only passive bystanders but more essential players in the inflammatory response. Current knowledge about the role of platelets in inflammation, infection, and immunity has been summarized by numerous recent

comprehensive reviews (Klinger and Jelkmann 2002; Weyrich et al. 2003; Weyrich and Zimmerman 2004; Elzey et al. 2005; Gawaz et al. 2005; Fitzgerald et al. 2006; Zarbock et al. 2007; Menezes et al. 2009; Smyth et al. 2009; Leslie 2010; Semple and Freedman 2010; Yeaman 2010; Nurden 2011; Semple et al. 2011; Huang and Chang 2012; Li et al. 2012; Projahn and Koenen 2012; Vieira-de-Abreu et al. 2012; Engelmann and Massberg 2013; Garraud et al. 2013; Ioannou et al. 2013; Jenne et al. 2013a; Mantovani and Garlanda 2013; Rondina et al. 2013; Verschoor and Langer 2013; Herter et al. 2014; Morrell et al. 2014; Rondina and Garraud 2014; Speth et al. 2014; Weyrich 2014; Yeaman 2014; Chabert et al. 2015; Garraud and Cognasse 2015; Hamzeh-Cognasse et al. 2015; Jenne and Kubes 2015; Lam et al. 2015; Thomas and Storey 2015).

Platelet contribution to immunity has been evaluated both in innate and adaptive responses. In sterile inflammation, platelets were studied in models of non-evolutionary, iatrogenic, or lifestyle-induced sterile injury, wherein dysregulated inflammation leads to inappropriate injury. This includes conditions like atherosclerosis, ischemia/reperfusion as happens in myocardial infarction, bowel ischemia, acute liver injury, or post liver transplantation, acute lung injury, fatty liver disease, and autoimmune diseases (Esch et al. 2010; Habets et al. 2013; Kohler et al. 2011a, b; Langer et al. 2012; Lievens and von Hundelshausen 2011; Salter et al. 2001; Zaldivar et al. 2010; Zarbock and Ley 2009). All of these conditions are caused by bad diet, clinical interventions, or toxicity and are not related to healthy repair. Under these dysregulated conditions, blocking either neutrophils or platelets leads to an improved outcome (Kohler et al. 2011a; Liu et al. 2011; Rossaint et al. 2014; Salter et al. 2001; Zarbock et al. 2006). Platelets also contribute to pathogenesis of autoimmune diseases, like rheumatoid arthritis, systemic lupus erythematosus, and multiple sclerosis, wherein dysregulated immunity is again a key feature (Boilard et al. 2010, 2012; Langer et al. 2012). Recent discoveries about platelet involvement in adaptive immune responses suggest that platelets might link innate and adaptive responses (Elzey et al. 2005; Morrell et al. 2014).

Although very poorly understood, platelet-neutrophil interactions in traumatic injury seem to be essential for successful healing (Slaba et al. 2015). In bacterial, viral, and parasitic infections, platelets have been shown to be an indispensable part of host defense. They contribute to elimination of pathogens by signaling to other immune cells as well as by direct platelet-derived defensive mechanisms, including direct bacterial killing (Jenne and Kubes 2015; Klinger and Jelkmann 2002; Yeaman 2010). It is becoming evident that platelets are not only linking hemostasis and thrombosis with inflammation and wound healing (Gawaz and Vogel 2013; Nurden 2011; Zarbock et al. 2007), but they are also involved in immune responses very broadly. Platelets may very likely be the most versatile effector cells in the immune continuum (Vieira-de-Abreu et al. 2012).

Sensing Inflammatory Stimuli

Pathogen Recognition Receptors Expressed by Platelets

In infection and sterile inflammation pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) are sensed by immune cells via pathogen recognition receptors (PRRs) (Kono and Rock 2008). Sensing these stimuli is the first step of the immune response during inflammation. Platelets express several types of PRRs—toll-like receptors (TLRs) 1, 2, 3, 4, 5, 6, 7, 9, CD14, CD40, and formyl peptide receptor (FPR) (Semple and Freedman 2010). TLRs represent a key family of inflammatory receptors binding a wide variety of ligands from both pathogens and damaged tissue. The discovery of TLRs on platelets represents an important milestone in understanding the role of platelets in inflammation and immunity. Messenger ribonucleic acid (mRNA) and protein levels of TLRs, as well as functional responses of platelets to TLR ligands, have been reported (Anabel et al. 2014; Andonegui et al. 2005; Aslam et al. 2006; Cognasse et al. 2005, 2008, 2015; Shiraki et al. 2004; Stahl et al. 2006; Ward et al. 2005; Zhang et al. 2009). Expression of TLRs on the platelet surface is mainly constitutive, although some TLRs, like TLR2 and 9, are upregulated upon platelet activation (Aslam et al. 2006). TLR4 has been the most studied toll-like receptor on platelets. Andonequi et al. first demonstrated that platelet TLR4 contributed to neutrophil-dependent platelet sequestration to the lung vasculature in response to lipopolysaccharide (LPS) (Andonegui et al. 2005). Their findings were extended by Aslam et al., who showed that platelet binding to LPS via TLR leads to thrombocytopenia and tumor necrosis factor alpha (TNFα) production (Aslam et al. 2006). In fact, platelets were shown to express all components of the LPS receptor signaling pathway, including TLR4, CD14, MD2, and MyD88. Although some investigators have reported that LPS leads to P-selectin expression, platelet aggregation, and mediator secretion (Zhang et al. 2009), others have found that LPS induces a different platelet phenotype. For example, engagement of platelet TLR4 was shown to cause platelet binding to neutrophils, without platelet aggregation, with subsequent release of NETs—a robust mechanism to ensnare bacteria in septic blood (Clark et al. 2007; Ma and Kubes 2008). Numerous groups have shown that exposure of platelets to pathogens or engagement of PRRs on the platelet surface has

many functional consequences including increase in intracellular calcium, platelet adhesion to collagen, platelet aggregation, glycoprotein IIb/IIIa (GPIIbIIIa) expression, and cytokine release (Aslam et al. 2006; Blair et al. 2009; Clawson and White 1971; Cognasse et al. 2008; Matera et al. 1992; Zhang et al. 2009; Zhu et al. 2012). Interestingly, platelets were shown to discriminate between various external signals through a single type of PRR and adjust their response by releasing different cytokines (Berthet et al. 2012). Besides TLR and LPS receptor signaling, thrombin-activated platelets were demonstrated to express functional FPR on their surface, and binding of *N*-formylated peptides mobilized calcium pools and induces platelet chemotaxis (Czapiga et al. 2005). However, it is important to recognize that a lot of studies use supraphysiological concentrations of stimuli raising issues of relevance.

Release of Inflammatory Mediators

Secretion of inflammatory mediators is an important mechanism by which platelets deliver signals to leukocytes, monocytes, endothelial cells, and other target cells. Platelet granules store over 1000 proteins, peptides, biologically active lipids, and eicosanoids, which are translocated to the platelet membrane and released upon platelet activation (Zufferey et al. 2012). Many of these substances are pro-inflammatory and contribute to intercellular signaling and inflammatory response at the local and systemic level (Weyrich and Zimmerman 2004). The capacity of the platelets to synthesize lipid mediators such as thromboxane A_2 (TXA_2) and platelet-activating factor (PAF) has been known for many years. However, the signal-dependent translation of interleukin-1 beta (IL-1β) by platelets has only recently been discovered (Freedman 2008; Lindemann et al. 2001; Weyrich et al. 2009; Weyrich and Zimmerman 2004).

Platelet Secretome

Platelets contain several types of granules with a number of important molecules released upon activation (Jurk and Kehrel 2005; Rendu and Brohard-Bohn 2001). Platelet dense bodies (δ-granules) contain strong amplifiers of platelet hemostatic response such as the nucleotides, adenosine triphosphate (ATP) and adenosine diphosphate (ADP), amines, bivalent cations, histamine, and serotonin, the latter two also being involved in monocyte and T cell signaling (Dale et al. 2002; Gachet 2001; Leon-Ponte et al. 2007; Nesbitt et al. 2003; Soga et al. 2007a, b). Platelet α-granules contain adhesion molecules such as von Willebrand factor (vWf), thrombospondin-1 (TSP-1), vitronectin, fibronectin, platelet-endothelial adhesion molecule-1 (PECAM-1, CD31) and GPIIbIIIa, mitogenic factors (platelet-derived growth factor (PDGF)), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β)), coagulation factors (V, VII, XI, XIII, fibrinogen, plasminogen, protein S), protease inhibitors (C1, plasminogen activator inhibitor 1 (PAI-1), tissue factor pathway inhibitor (TFPI)), and chemokines (CCL3, 5, 7, 17 and CXCL1, 4, 5, 7, 8) (Blair and Flaumenhaft 2009). Recently, key proteins of the major histocompatibility complex class I (MHC class I) antigen-presenting pathway, such as β2-microglobulin, 26S protease regulatory subunit 10B from the proteasome, and proteins 1 and 2 from the transporter associated with antigen processing, were identified in α-granules (Zufferey et al. 2014). Platelet α-granules are diversified into distinct populations which allows differential release of active molecules in response to various stimuli (Italiano et al. 2008; Sehgal and Storrie 2007). Molecules in α-granules are either soluble or membrane bound being exposed on the platelet membrane after exocytosis. Many of the α-granule proteins are also constitutively expressed on platelet membranes, including GPIIbIIIa, glycoprotein VI (GPVI), PECAM, and glycoprotein Ib-V-IX complex (GPIb-V-IX). Others, such as P-selectin, are exclusively expressed in α-granules, being exposed on the platelet surface only after platelet activation (Maynard et al. 2007). Platelet lysosomes contain proteases, glycosidases, and cationic proteins with bactericidal activity (Rendu and Brohard-Bohn 2001). Recent work suggests that at least some of the platelet proteins are systematically stored and organized within the granules in the form of sub-granule vesicles or microparticles, each microparticle containing one specific protein (Zhang and Yang 2012).

Upon activation, platelets secrete a number of potent procoagulant, pro-inflammatory, and mitogenic substances which modulate functions of other platelets, leukocytes, and endothelial cells: chemokines, cytokines (IL-1β, CD40L, β-thromboglobulin (βTG)), growth factors, and coagulation factors. These mediators participate in cell survival, proliferation, coagulation and fibrinolysis, chemotaxis, and cell adhesion (Zarbock et al. 2007). Platelet mediators secreted in soluble form act mainly in a paracrine or in an endocrine manner. However, some of the mediators act in a juxtacrine manner—retained on the platelet membrane bound to receptor and available for intercellular signaling (Vieira-de-Abreu et al. 2012).

CXC chemokines secreted by platelets are represented by platelet factor 4 (PF4, CXCL4), βTG (CXCL7), and interleukin 8 (IL-8, CXCL8). CXC chemokines induce chemotaxis of neutrophils, although more importantly, when secreted from platelets immobilized to the vessel wall, together with rapidly synthesized PAF, they trigger activation of rolling neutrophils and their firm adhesion to

endothelial cells via β_2 integrins (Brandt et al. 2000; McIntyre et al. 2003). PF4 and βTG are recognized by CXC receptors 1 and 2 on neutrophils, and they might provide both stimulatory and inhibitory effects (Boehlen and Clemetson 2001; Brandt et al. 2000; Elstad et al. 1995; McIntyre et al. 2003).

CC chemokines enhance paracrine activation of platelets and increase activation of neutrophils (Brandt et al. 2000; Cheng et al. 2001). CCL5 (regulated on activation, normal T cell expressed and secreted, RANTES, or fractalkine) not only activates platelets but also directly targets many other inflammatory cells—neutrophils, monocytes, eosinophils, basophils, natural killer (NK) cells, T lymphocytes, and dendritic cells (DCs). RANTES secreted and deposited by adherent platelets or platelets associated with monocytes in heterotypic aggregates promotes adhesion of monocytes to the endothelium, their activation, and chemokine and cytokine secretion (Schober et al. 2002; Weyrich et al. 1995, 1996). Macrophage inflammatory protein 1 alpha (MIP-1α, CCL3) which targets multiple immune cells—monocytes, eosinophils, basophils, NK cells, CD8+ T lymphocytes, and DC subsets—induces chemotaxis. It can also release histamine from basophils. Monocyte chemotactic protein 3 (MCP-3, CCL7) has similarly diverse effects (Baggiolini and Dahinden 1994; Taub et al. 1993; Weyrich et al. 1996; Weyrich and Zimmerman 2004). Interestingly, platelets display CXC and CC chemokine receptors (CXCR4, CCR1, CCR3, CCR4) on their surface which might suggest autocrine and/or bidirectional paracrine signaling (Boehlen and Clemetson 2001). A comprehensive and updated overview of platelet chemokines was recently published by Karshovska et al. (2013).

Platelet-associated cytokine CD40L (CD154) is a transmembrane protein of the tumor necrosis factor (TNF) family, recognized by CD40 receptor on B lymphocytes, monocytes, endothelial cells, and platelets. CD40L-CD40 complex is recognized as a major mechanism in immune regulation (Blumberg et al. 2009; Henn et al. 1998). Platelet-derived CD40L induces chemokine secretion and upregulation of adhesion molecules on endothelial cells, mediates immunoglobulin M (IgM) to immunoglobulin G (IgG) isotype switch in B cells and DC maturation, and augments T cell responses (Elzey et al. 2003; Henn et al. 1998). Some authors have also suggested reciprocal intercellular signaling of platelets and other cells expressing components of the CD40L-CD40 system (Danese et al. 2004).

The above highlights the huge diversity of the platelet secretome. Although all these factors are made by platelets, whether they are the primary source of these cytokines in blood in the context of inflammation remains controversial. However, unexpectedly high local concentrations of some of the chemokines surrounding activated and aggregated platelets suggest that platelets might be the primary source of these chemokines (Boehlen and Clemetson 2001). Under certain circumstances such as in patients with immune thrombocytopenia, platelets might be the principle source of released cytokines (Andersson et al. 2000; Semple et al. 2011; Stasi et al. 2008).

Platelet Transcriptome

In addition to secretion of the granule content, activated platelets have also synthetic capacities. The ability of activated platelets to synthesize lipid mediators such as TXA_2 and PAF is well known (Weyrich and Zimmerman 2004). However, their capacity to synthesize proteins was not recognized until recently (Weyrich et al. 2009). IL-1β, one of the most important regulators of inflammatory responses, is produced by activated platelets in a process described as a signal-dependent translation (Lindemann et al. 2001; Weyrich et al. 2003). IL-1β pre-messenger RNA (pre-mRNA) is incorporated into proplatelets by megakaryocytes during thrombopoiesis together with functional spliceosome (Denis et al. 2005). Upon platelet activation by platelet agonists, LPS, or engagement of platelet integrins, Fc receptors or TLR4, pre-mRNA is spliced into the mature IL-1β transcript and translated into the protein. IL-1β is secreted by platelets in both a paracrine and juxtacrine manner, incorporated into the fibrin mesh or shed in the form of IL-1β positive microparticles (Brown and McIntyre 2011; Denis et al. 2005; Lindemann et al. 2001; Qian et al. 2008; Shashkin et al. 2008). Synthesis and secretion of IL-1β by platelets was shown to be abolished by inhibition of $\alpha_{IIb}\beta_3$ integrin (GPIIbIIIa) suggesting direct linkage between hemostasis and inflammation (Lindemann et al. 2001). The presence of regulatory mechanisms of IL-1β secretion by platelets suggests that platelets are an important source of this cytokine (Denis et al. 2005; Lindemann et al. 2001).

Receptors for IL-1β are present on a large number of immune cells which makes secretion of IL-1β by platelets potentially a very important mechanism for the inflammatory response. The principal target cells for platelet-derived IL-1β are endothelial cells. IL-1β induces secretion of chemokines (CXCL8, CCL2), cytokines (IL-6, IL-8, epithelial neutrophil-activating protein 78 (ENA-78)), and endothelial adhesion molecules (E-selectin, vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), integrin $\alpha_v\beta_3$) leading to amplification and propagation of the inflammatory response by recruitment and activation of neutrophils and monocytes (Gawaz et al. 2000; Hawrylowicz et al. 1991). IL-1β represents a major danger signal for dendritic cells inducing their maturation and activation. Thus, local release of IL-1β by

platelets might represent an important signal to dendritic cells linking innate and adaptive immune responses (Gallucci and Matzinger 2001). It has recently been shown that platelets express interleukin-1 receptor 1 (IL-1R1) on which IL-1β acts as an agonist promoting its own synthesis through an autocrine stimulatory loop. Interestingly, this IL-1β autocrine loop was required to amplify platelet activation induced by LPS (Brown et al. 2013).

Recently, the platelet transcriptome has been under intensive investigation. Platelets were shown to contain a wide array of RNAs: messenger RNA (mRNA), transfer RNA (tRNA), microRNA (miRNA), long noncoding RNA, and, more recently, circular RNA (circRNA) (Alhasan et al. 2016; Bray et al. 2013; Edelstein et al. 2013). Surprisingly, this rich platelet transcriptome was found to have only a weak correlation with the platelet proteome (Londin et al. 2014). Platelet miRNA was proposed to be a regulatory mechanism of platelet mRNA and protein expression levels and the main source of circulating miRNA. Thus, circulating miRNA originating from platelets may serve as a biomarker of platelet function. Moreover, circulating miRNA may play an important role in modulating the function of other cells, particularly immune cells (Lindsay and Edelstein 2016; McManus and Freedman 2015). CircRNA was proposed to be a degradation product of the platelet transcriptome, although biological effects outside of platelets cannot be excluded (Alhasan et al. 2016; Provost 2016). However, while our knowledge of the platelet transcriptome has markedly evolved over the last several years, its significance in immunity has to be further elucidated.

Platelet-Derived Microparticles

Microparticles are small (<1 μm), spherical secretory organelles of lipid composition (Cocucci et al. 2009; Nomura et al. 2008). Microparticles are shed from erythrocytes, leukocytes, platelets, and endothelial cells. However, 80 % of circulating microparticles originate from platelets (Horstman and Ahn 1999). Platelet-derived microparticles are usually smaller than 500 nm, the main populations being 100–250 nm in size (Aatonen et al. 2014). Microparticles could be derived both from activated and nonactivated or apoptotic platelets (Cauwenberghs et al. 2006; Morel et al. 2011; Vasina et al. 2011). Platelet-derived microparticles were shown to be an important transport mechanism for platelet-secreted pro-inflammatory and prothrombotic molecules, including tissue factor (TF), CD40L, IL-1β, or RANTES (Boilard et al. 2010; Elzey et al. 2005; Mause et al. 2005; Zwicker et al. 2011). Some authors have recently demonstrated that platelet-derived microparticles might also transport transcription factors such as nuclear factor kappa B (NFkB) and peroxisome proliferator-activated receptor gamma (PPARγ) (Lannan et al. 2015). Moreover, platelet microparticles could deliver platelet miRNA to target cells, especially endothelial cells and possibly other cells, such as neutrophils, and therefore alter gene expression in those cell types (Laffont et al. 2013; Lindsay and Edelstein 2016; Semple 2013). Detailed proteomic analysis has revealed that platelet activation pathways determine the number and the content of formed microparticles (Aatonen et al. 2014). A recent study showed that microparticles might not only represent a transport mechanism but also a storage system within platelet granules (Zhang and Yang 2012).

Platelets Recruit Immune Cells

Platelets possess a wide repertoire of adhesion molecules, β1 and β3 integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$ (GPIIbIIIa), and $\alpha_v\beta_3$), membrane glycoproteins (GPIb-V-IX, GPIIbIIIa ($\alpha_{IIb}\beta_3$ integrin), GPVI), selectins (P-selectin and P-selectin glycoprotein ligand 1, PSGL-1), and vWf, which allows them to directly interact with endothelial cells, neutrophils, and monocytes (Bennett 2005; Bennett et al. 2009; Kansas 1996; Ruggeri 2003; Zarbock et al. 2007).

Platelets Recruit Neutrophils

It has been well known for many years that platelets can recruit neutrophils to the endothelium via so-called secondary capture. Neutrophil rolling, firm adhesion, and transmigration are distinct steps in the recruitment process. Platelets adherent to the endothelium express P-selectin which tethers neutrophils to the vessel wall. Neutrophil rolling on adherent platelets is mediated by interaction of platelet P-selectin with neutrophil PSGL-1 (Yang et al. 1999). The production of PAF activates integrins on neutrophils and allows them to firmly adhere to immobilized platelets (McIntyre et al. 2003; Ostrovsky et al. 1998; Weber and Springer 1997). The integrins involved include macrophage-1 antigen (Mac-1, CD11b/CD18) on neutrophils and GPIIbIIIa or GPIbα on platelets, although some authors have reported that lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) which binds to intercellular adhesion molecule 2 (ICAM-2) on platelets also plays a role (Diacovo et al. 1994, 1996; Evangelista et al. 1996; McIntyre et al. 2003; Piccardoni et al. 1996; Simon et al. 2000; Weber and Springer 1997; Zarbock et al. 2007). This mechanism of neutrophil recruitment has been demonstrated in numerous diseases including acute lung injury, ischemia reperfusion in the bowel and kidney, autoimmune glomerulonephritis, and cholestatic hepatitis. Platelet depletion, as well as P-selectin and GPIIbIIIa blockade, significantly decreased the number of

neutrophils recruited to the site of injury and improved outcome of a dysregulated immune response (Devi et al. 2010; Laschke et al. 2008; Salter et al. 2001; Singbartl et al. 2000; Zarbock and Ley 2009; Zarbock et al. 2006, 2007).

Platelets Recruit Monocytes

Similarly, platelets adherent to the endothelium recruit circulating monocytes through P-selectin/PSGL-1 interactions. Secretion and deposition of RANTES promote firm adhesion of monocytes (Kuijper et al. 1998; Schober et al. 2002; von Hundelshausen et al. 2001; Weyrich et al. 2002). Integrins involved in firm adhesion of monocytes to platelets involve Mac-1 on monocytes and GPIbα on platelets, although some authors have suggested that other receptors—ICAM-2, junctional adhesion molecule 3 (JAM-3), and GPIIbIIIa via fibrinogen bridges—might also play a role (Diacovo et al. 1994; Santoso et al. 2002; Simon et al. 2000). The exact importance of each of these molecules remains to be delineated, and this is not trivial as this mechanism of monocyte recruitment by platelets has been described in atherosclerosis and vascular restenosis (Gawaz et al. 2005; Lievens and von Hundelshausen 2011; Wang et al. 2005).

Circulating Heterotypic Platelet Aggregates

Activated platelets could adhere to leukocytes in circulating blood via P-selectin/PSGL-1 interactions, forming heterotypic aggregates. Circulating heterotypic platelet-neutrophil and platelet-monocyte aggregates were demonstrated in many diseases. These include, but are not limited to, atherosclerosis, acute coronary syndromes, acute cerebral ischemia, diabetes, smoking, thromboembolic disease, cystic fibrosis, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, myeloproliferative diseases, sepsis, acute lung injury, dengue, and post renal transplant (Smyth et al. 2009; Vieira-de-Abreu et al. 2012; Weyrich and Zimmerman 2004). Some authors consider formation of platelet-monocyte aggregates in vivo the most sensitive marker of platelet activation (Michelson et al. 2001), although their distinct role is not yet fully understood. Platelets in heterotypic aggregates induce activation of leukocytes including expression of β_2 integrins and release of chemokines, such as monocyte chemoattractant protein 1 (MCP-1, CCL2) and cytokines (IL-1β, IL-8, TNFα) (Weyrich et al. 1995, 1996). Others have demonstrated that monocytes engaged in circulating heterotypic platelet aggregates transmigrate more easily (da Costa Martins et al. 2006).

Platelets Are Recruited by Both Neutrophils and Endothelium

Platelets Are Recruited by Neutrophils

It has been known for many years that platelet sequestration to inflamed tissues is neutrophil and P-selectin dependent (Andonegui et al. 2005; Bednar et al. 1985; Hamburger and McEver 1990; Issekutz et al. 1983). However, the exact mechanism of platelet recruitment by neutrophils was not identified. Recently, it has been shown that neutrophils recruited to inflamed vessels extend a PSGL-1-bearing microdomain in the uropod into the vessel lumen that scans for activated platelets present in the bloodstream through P-selectin. Interaction of platelet P-selectin with the neutrophil uropod-localized cluster of PSGL-1 is essential for neutrophil chemotaxis and further propagation of inflammation (Jenne et al. 2013b; Sreeramkumar et al. 2014). In addition, the binding of platelets by neutrophils also appears to induce NET formation, although under these conditions P-selectin may not be the dominant adhesion molecule. Indeed, McDonald et al. reported an important role for LFA-1, but not P-selectin, in bacterial-induced platelet recruitment by neutrophils (McDonald et al. 2012). By contrast, virally induced platelet recruitment by neutrophils appeared to be more dependent upon Mac-1 and CD41 (integrin αIIb, GPIIb) (Jenne et al. 2013b).

Platelets Are Recruited by Inflamed Endothelium

Both activated and resting platelets can be recruited to the inflamed endothelium, and several different mechanisms have been described. Activated endothelial cells express P-selectin to which platelets bind via the GPIb-V-IX complex or PSGL-1 (Frenette et al. 2000; Romo et al. 1999). Alternatively, activated platelets expressing P-selectin adhere to endothelial PSGL-1 (da Costa et al. 2007). Some authors have shown that vWf could also play an important role in platelet adhesion to inflamed endothelium (Etingin et al. 1993). Others still have demonstrated that platelets are recruited to the inflamed endothelium through GPIIbIIIa/fibrinogen interaction with ICAM-1, eventually in cooperation with GPVI (Devi et al. 2010; Khandoga et al. 2002; Massberg et al. 1999). Recently, a novel mechanism of platelet recruitment to the inflamed endothelium was described by Meyer Dos et al. who demonstrated that flowing platelets could be captured, activated, and immobilized directly on endothelial chemokine CXCL16 in atherosclerotic plaques (Meyer Dos et al. 2015). This firm platelet adhesion through GPIIbIIIa leads to expression of CD40L (CD154) and IL-1β by platelets which induce a

pro-inflammatory phenotype of the endothelium. Endothelial cells release chemokines (MCP-1) and cytokines (CD40L, IL-6, IL-8, TF), express adhesion molecules (E-selectin, P-selectin, VCAM-1, ICAM-1, αvβ3), and interact with leukocytes further propagating the inflammatory response (Gawaz et al. 2000; Hawrylowicz et al. 1991; Henn et al. 1998; Kaplanski et al. 1993; McIntyre et al. 2003).

Platelets in Sterile Inflammation

In sterile inflammation, the immune system is activated by nonmicrobial signals. Damaged or dead cells liberate a plethora of endogenous molecules, recognized by the innate immune system as danger signals, DAMPs, or alarmins. Immune cells sense alarmins through PRRs which translates to generation of the IL-1β inflammasome and downstream propagation of the inflammatory response by neutrophils, monocytes/macrophages, and endothelial cells (Chen and Nunez 2010; Kono and Rock 2008; McDonald and Kubes 2011). Platelets were not considered an integral part of sterile inflammatory responses until recently. However, there is now a growing body of evidence that platelets propagate and/or modulate the inflammatory response of other immune cells, although their distinct role in sterile inflammation is not yet fully clarified (Fig. 1).

Platelets express several functional PRRs (TLR1, 2, 3, 4, 5, 6, 7, and 9, CD14, CD40, and FPR) which gives them a capacity to sense danger signals (alarmins) (Andonegui et al. 2005; Aslam et al. 2006; Czapiga et al. 2005; Semple and Freedman 2010). Engagement of these receptors was shown to cause platelet activation, adhesion, aggregation, and P-selectin expression during infection (Aslam et al. 2006; Blair et al. 2009; Clawson and White 1971; Cognasse et al. 2008; Matera et al. 1992). However, there is only limited evidence that platelets are able to sense and become directly activated by DAMPs. As reviewed by Kono and Chen, the following are the signals which could be sensed by the above receptors expressed on platelets: high-mobility group box 1, HMGB-1 (TLR2, 4, and 9); heat shock proteins, HSPs (TLR2, TLR4, CD14, CD40); ribonucleic acid, RNA (TLR3); deoxyribonucleic acid, DNA (TLR9); uric acid (TLR2, 4, CD14); *N*-formylated peptides (FPR-1, FPR-L1); defensins (TLR4); hyaluronan (TLR2, 4); biglycan (TLR2, 4); versican (TLR2); heparan sulfate (TLR4); and IL-1α (IL-1R) (Chen and Nunez 2010; Kono and Rock 2008). To date, only some of these danger signals were shown to be sensed by platelets with functional consequences. Sensing HMGB-1 via platelet TLR4 was documented to cause platelet aggregation through GPIIbIIIa (Yang et al. 2015), while other authors have demonstrated that HMGB-1 binds to activated platelets via receptor for advanced glycation end products (RAGE) and have suggested a potential role for this mechanism in atherothrombosis (Ahrens et al. 2015). Activated platelets were also shown to present HMGB-1 to neutrophils promoting NET formation and autophagy (Maugeri et al. 2014). Engagement of TLR4 ligands on platelets resulted in differential release of various cytokines, including RANTES, CD40L, and PF4 (Cognasse et al. 2008). Binding of *N*-formyl peptide to the surface of activated platelets resulted in platelet chemotaxis (Czapiga et al. 2005). Other authors showed that advanced glycation end products (AGEs), substances which are considered worsening factors in many chronic sterile inflammatory diseases, like diabetes and atherosclerosis, bind to platelet CD36 inducing prothrombotic and pro-inflammatory phenotypes (Zhu et al. 2012). On the other hand, uric acid did not affect platelet activation and aggregation (Ciompi et al. 1983), and some of the HSPs were demonstrated to even inhibit platelet activation and aggregation (Matsuno et al. 1998; Tsujimoto et al. 2015). It seems that platelets are able to sense several alarmins, many of which directly activate platelets, while others have no effect or even inhibit platelet functions. This means that platelets might be involved in sterile inflammation in different ways. However, activation of platelets with some danger signals leads to an increase in aggregation and secretion of pro-inflammatory mediators, resulting in paracrine activation and direct interaction with other immune cells. However, whether platelets further propagate or regulate immune responses to sterile inflammation remains unclear (Duerschmied et al. 2014; Maugeri et al. 2012; Morrell et al. 2014; Projahn and Koenen 2012; Rondina and Garraud 2014).

The role of platelets in sterile inflammation has been studied in multiple diseases, including atherosclerosis, acute lung injury, ischemia/reperfusion, and autoimmune disorders. Their role was demonstrated mostly as pro-inflammatory, and modulation of platelet activation in these diseases often improved outcome (Boilard et al. 2012; Gawaz et al. 2005; Langer et al. 2012; Lievens and von Hundelshausen 2011; Salter et al. 2001; Singbartl et al. 2000; Zarbock and Ley 2009; Zarbock et al. 2006).

Platelets in Atherosclerosis and Metabolic Diseases

In atherosclerosis platelets were shown to be critical in the induction of vascular wall inflammation leading to the development of atherosclerotic lesions and so-called atherothrombosis. The evolution of atherosclerotic plaques starts with activated platelets which roll (via selectins) and subsequently firmly adhere (via β3 integrins) to endothelial cells. Secretion of CD40L and IL-1β inflames the

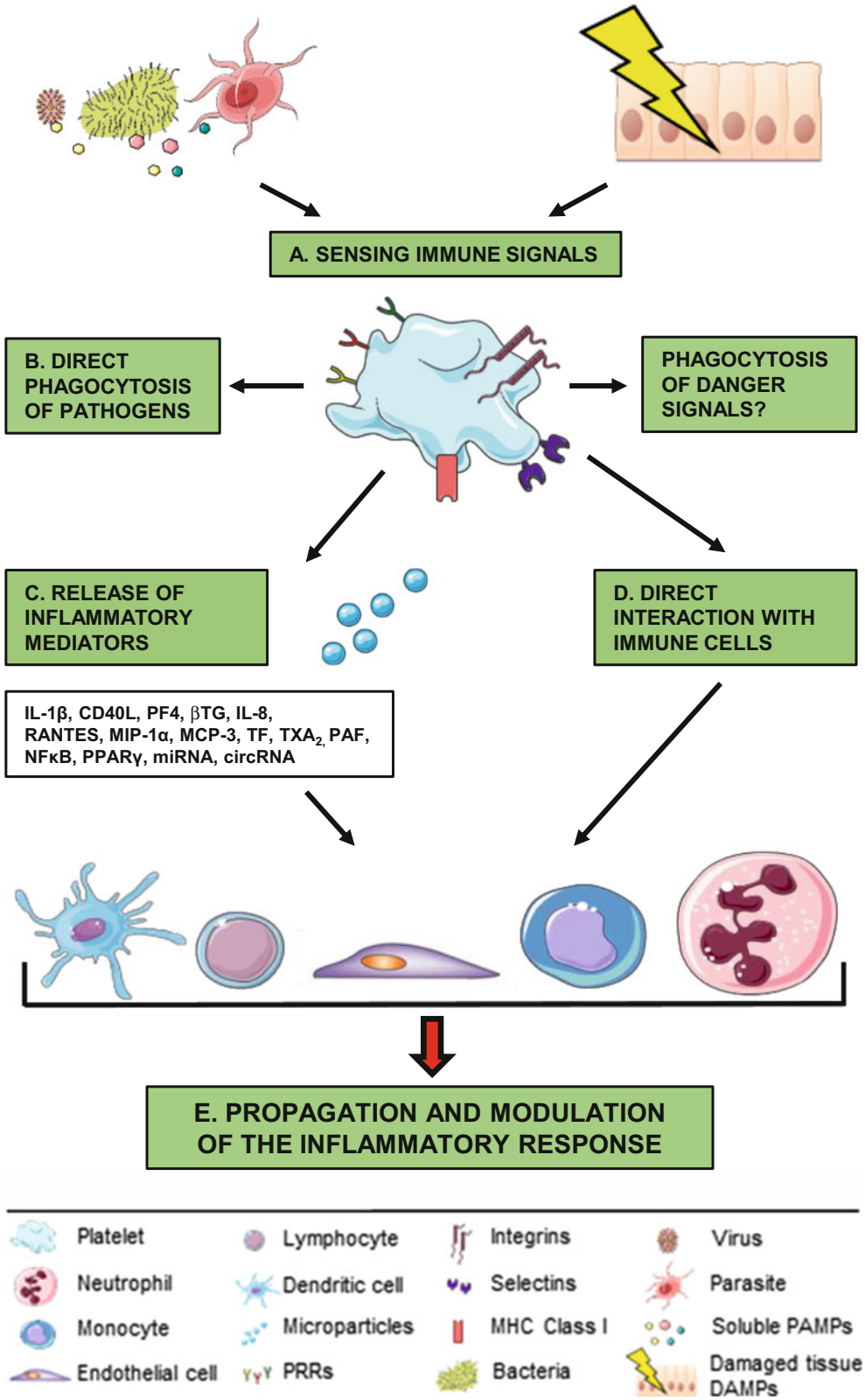

Fig. 1 Platelets and the immune system. (**A**) Platelets sense immune signals. Platelets express pathogen recognition receptors, like TLRs, CD40, and FPR, allowing them to sense immune signals derived from pathogens (PAMPs) as well as damaged tissue (DAMPs). (**B**) Direct phagocytosis of pathogens. In response to pathogens, platelets are directed to sites of infection, cluster around the microorganisms, and directly phagocytose them. It is not clear whether platelets phagocytose also parts of damaged tissues. (**C**) Release of pro-inflammatory

endothelium. Subsequently, platelets recruit circulating leukocytes (through deposition of RANTES and adhesion via selectins and β_2 and β_3 integrins) and facilitate their transmigration and foam cell formation (Huo et al. 2003; Massberg et al. 2002). Badrnya et al. recently described that forming of heterotypic platelet-monocyte aggregates in hyperlipoproteinemia facilitates uptake of low-density lipoprotein by monocytes and their extravasation and foam cell formation (Badrnya et al. 2014). Platelets are understood to be central orchestrators of atherosclerotic plaque formation, and antiplatelet therapy has become a mainstay in the treatment of cardiovascular and cerebrovascular diseases (Coutts et al. 2015; Gawaz et al. 2005; Lievens and von Hundelshausen 2011; Tanguay et al. 2013). Based on a close interplay between sterile inflammation and thrombosis, atherosclerosis has been quoted as a thromboinflammatory disease (Nagareddy and Smyth 2013; Nieswandt et al. 2011). Similarly, metabolic syndromes such as obesity and diabetes mellitus have sterile inflammation as a common denominator. Loss of platelet inhibition by insulin leads to platelet activation, increased expression of P-selectin and GPIIbIIIa on the platelet surface, and the systemic thromboinflammatory state (Anfossi et al. 2009; Morange and Alessi 2013). It was suggested that platelet signaling to monocytes and macrophages links leukocyte activation, cytokine production, and insulin resistance together in these syndromes (Gustafson et al. 2007; Neels and Olefsky 2006).

Platelets in Acute Lung Injury (ALI)

The interplay between platelets, neutrophils, and endothelial cells was demonstrated to be critical in the etiology of acute lung injury. Activated platelets form heterotypic platelet-neutrophil aggregates and induce sequestration of neutrophils to the pulmonary vasculature. Interaction of platelets and neutrophils with endothelial cells induces secretion of TXA_2 which has detrimental effects on vascular permeability and oxygenation. Blocking either platelets (using a P-selectin antibody or platelet depletion) or neutrophils dramatically improved gas exchange and survival. Although these authors stated that the precise mechanism of the role of platelets for neutrophil recruitment into the inflamed lungs remained to be elucidated, it seemed very compelling that platelets were key modulators of sterile inflammatory response in ALI (Zarbock et al. 2006, 2007).

Platelets in Ischemia/Reperfusion Injury

Similarly to ALI, a platelet-neutrophil-endothelium interplay was shown to be central in the pathogenesis of mesenteric ischemia/reperfusion injury. Neutrophil recruitment into postischemic venules and their emigration to tissues were shown to be platelet dependent. Blocking either P-selectin, GPIIbIIIa, or fibrinogen significantly reduced the number of adherent and emigrated leukocytes. Indeed, platelets seemed to have a pivotal role in modulating the inflammatory response to sterile injury in bowel ischemia (Salter et al. 2001). A central role of neutrophils, endothelium, and platelets was also suggested in a model of ischemia-/reperfusion-induced acute renal failure. Authors demonstrated that blocking P-selectin significantly decreased neutrophil recruitment and prevented onset of acute renal failure. However, the contribution of platelet P-selectin versus endothelial P-selectin remained to be clarified in this model (Singbartl et al. 2000). Platelet-neutrophil complexes were shown to play an important role both in myocardial and liver ischemia/reperfusion injury.

mediators. Engagement of PRRs on platelet surface leads to platelet activation, adhesion, aggregation, and release of pro-inflammatory and procoagulant molecules. These include, but not limited to, cytokines (IL-1β, CD40L, IL-8), chemokines (RANTES, PF4, MIP-1α, MCP-3, βTG), procoagulant molecules (TF, TXA_2, PAF), and nuclear factors (NFkB, PPARγ). Some of these molecules, like IL-1β, TF, CD40L, RANTES, NFkB, PPARγ, and miRNA, could be stored and released in the form of microparticles. Defensins, thrombocidins, and kinocidins secreted by platelets have direct bactericidal and fungicidal properties. (**D**) Direct interaction with immune cells. Platelets express a repertoire of selectins, β1 and β3 integrins, and membrane glycoproteins which allows them to directly interact with immune cells. Platelets adherent to the vascular wall recruit neutrophils and monocytes via so-called secondary capture. Platelet P-selectin tethers leukocytes to the vascular wall, followed by rolling via P-selectin/PSGL-1 and subsequent firm adhesion through integrins. Adhesion molecules include Mac-1 and LFA-1 on leukocytes and GPIIbIIIa, GPIbα, or ICAM-2 on platelets. Platelets and leukocytes also form circulating heterotypic aggregates which are considered to be a sign of platelet activation and promote activation of neutrophils and monocytes and their recruitment to tissues. Platelet interaction with neutrophils induces NET formation allowing to entrap pathogens in flowing blood. Firm platelet adhesion via GPIIbIIIa and secretion of CD40L and IL-1β induces pro-inflammatory phenotype of the endothelium, release of chemokines (MCP-1) and cytokines (CD40L, IL-6, IL-8, TF), and expression of adhesion molecules (E-selectin, P-selectin, VCAM, ICAM, αvβ3). Similarly, platelets themselves can be recruited by leukocytes and inflamed endothelium. Recently, platelets were demonstrated to be involved in T cell responses and induce B cell isotype switch, dendritic cell maturation, and antigen presentation. (**E**) Propagation and modulation of the inflammatory response. A wide array of pro-inflammatory mediators and direct interactions with immune cells lead to global activation of the immune system and further propagation and modulation of the inflammatory response. Platelets were shown to contribute to systemic inflammation in many sterile inflammatory diseases, to be indispensable in elimination of pathogens, and to link innate and adaptive responses together

Vasodilator-stimulated phosphoprotein (VASP), a central cytoskeleton protein affecting actin dynamics, was identified as a key regulator of platelet-neutrophil complex formation during the reperfusion phase, and inhibition of VASP significantly reduced formation and deposition of platelet-neutrophil complexes, as well as the extent of ischemia/reperfusion injury (Kohler et al. 2011a, b). Similarly, platelet activation and their interaction with monocytes played an important role in early graft dysfunction caused by reperfusion injury subsequent to cold ischemia in orthotopic liver transplantation (OLT) (Esch et al. 2010).

Platelets in Autoimmune Diseases

A significant contribution of platelets has been suggested to be involved in several autoimmune diseases, particularly in multiple sclerosis (MS), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (Habets et al. 2013). In experimental autoimmune encephalitis (EAE), a model of MS, platelets were present in MS lesions and promoted central nervous system inflammation. Neutrophil recruitment to the brain lesions was demonstrated to be platelet dependent, and attenuation of platelets and their receptors (GPIbα and GPIIbIIIa) significantly reduced severity of the disease (Langer et al. 2012). Neutrophil recruitment to the MS brain lesions could be promoted by platelet-derived serotonin, and treatment of MS patients with selective serotonin reuptake inhibitors (SSRI) also reduced severity of the disease (Duerschmied et al. 2013; Hofstetter et al. 2005).

Different mechanisms of platelet involvement were described in RA and SLE. In RA, platelets and platelet-derived proteins were shown to be present in the synovium and synovial fluid in higher amounts, and serum levels of platelet-derived P-selectin and CD40L correlated with disease activity (Ertenli et al. 1998; Knijff-Dutmer et al. 2002; Tamura et al. 2001). Recently, Boilard et al. demonstrated that platelet production of IL-1β positive microparticles inflamed synovial fibroblasts which were central to joint inflammation. The production of IL-1β-positive microparticles from platelets was triggered by stimulation of collagen receptors, GPVI. Platelet-derived serotonin and prostaglandins further increased vascular permeability and synovial inflammation (Boilard et al. 2010).

In SLE, platelets are activated by circulating immune complexes through their FcγRIIA receptor. Activated platelets signal plasmacytoid dendritic cells through CD40L-CD40 juxtacrine interaction which leads to secretion of IFNα production by dendritic cells. Platelet depletion or inhibition of P2Y12 receptor decreased severity of the disease and improved overall survival (Boilard et al. 2012; Duffau et al. 2010). Platelets also played an important role in lupus nephritis, where they induced proliferation of mesangial cells and production of profibrotic TGF-β (Delmas et al. 2005).

As a constant pool of circulating pro-inflammatory mediators and now recognized as sentinel immune cells, platelets are readily available for intercellular interactions able to contribute to many other autoimmune diseases as recently suggested in systemic sclerosis and Graves' disease (Kuznik et al. 2014; Ramirez et al. 2012).

Platelets in Localized Sterile Injury

Contrary to systemic sterile inflammatory diseases like atherosclerosis, ischemia/reperfusion, or autoimmune diseases where platelets have been demonstrated to have detrimental pro-inflammatory effects, in a localized sterile injury such as that occurs following trauma or burns, platelets were shown to be beneficial to the host by augmenting neutrophil recruitment to allow subsequent wound repair (Slaba et al. 2015). It is intriguing that so few studies are available in the area of platelets and sterile injury and repair, considering that platelets are used clinically to help in repair (Lacci and Dardik 2010; Toscano and Holtzclaw 2008).

Platelets in Infection

In infection PAMPs are detected by innate immune cells via PRRs. Pathogens are eliminated by a coordinated innate immune response, followed by adaptive immune responses (Janeway Charles et al. 2001). The role of platelets in antimicrobial host defense has been intensively studied, and it is becoming quite well understood. Platelets are targeted to the sites of infection and directly interact with bacteria. They become activated and contribute to the host defense by direct clearance of pathogens and by signaling to other immune cells (Jenne and Kubes 2015; Semple and Freedman 2010; Semple et al. 2011; Yeaman 2010, 2014).

Platelets Are Targeted to Sites of Infection and Directly Interact with Pathogens

The evidence that platelets are activated by pathogens and PAMPs has been documented by many authors. Platelets were shown to express TLRs (1, 2, 4, 6, and 9), and their engagement or stimulation of platelets with bacterial products (LPS) or bacteria themselves leads to platelet activation (P-selectin and GPIIbIIIa expression), adhesion, aggregation, secretion of CD40L and PAF, formation of platelet-neutrophil heterotypic aggregates, and production of TNFα by macrophages (Aslam et al. 2006; Blair et al. 2009; Clawson and White 1971; Cognasse et al. 2005, 2008;

Matera et al. 1992; Zhang et al. 2009). Furthermore, it has been shown that platelets could be directly activated by viruses through TLR7 and engagement of TLR3 induced platelet-neutrophil interaction and NET formation (Jenne et al. 2013b; Koupenova et al. 2014). Activated platelets were directed to the sites of infection, platelets formed clusters around microorganisms, encapsulated them, and facilitated their clearance (Calderone et al. 1978; Ferguson et al. 1986; Herd and Page 1995; Scheld et al. 1978; Wong et al. 2013; Yeaman 2010). Platelets have also been shown to rapidly accumulate at the sites of vascular infections, like infective endocarditis, thrombophlebitis, catheter site infections, vascular prosthesis, and stents (Ferguson et al. 1986; Scheld and Sande 1995; Scheld et al. 1978; Vinter et al. 1984). More recent work has suggested that platelets are able to migrate to extravascular space where they can affect infections (Pitchford et al. 2008). Direct contact of platelets with bacteria, viruses, and parasites may induce phagocytosis of the pathogen by platelets. For example, *S. aureus*, HIV, influenza, dengue, HCV, *P. vivax*, and *T. gondii* are all pathogens which have been shown to be phagocytosed by activated platelets (DaMatta et al. 1998; Danon et al. 1959; de Almeida et al. 2009; Fajardo 1979; Jaff et al. 1985; Movat et al. 1965; Noisakran et al. 2009; Youssefian et al. 2002). Platelets also produce several bactericidal and fungicidal proteins and peptides, defensins, thrombocidins (thrombin-induced platelet microbicidal proteins), and kinocidins (chemokines with microbicidal activity) allowing direct pathogen killing (Cole et al. 2001; Koo et al. 1996; Krijgsveld et al. 2000; Yang et al. 2003; Yeaman 2010, 2014). Moreover, platelet microbicidal proteins have synergistic effects and enhance antibiotic-induced bacterial killing (Mercier et al. 2004; Yeaman et al. 1992).

Platelets Contribute to Elimination of Pathogens by Signaling to Other Immune Cells

Platelets activated by interaction with pathogens signal to other immune cells through their secretome and direct intercellular interactions. Activated platelets secrete a number of chemokines attracting neutrophils as well as cytokines CD40L and IL-1β which inflame endothelial cells. Inflamed endothelial cells express an array of adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin, promoting neutrophil recruitment (Henn et al. 1998). Platelets can also promote neutrophil recruitment via so-called secondary capture where neutrophil rolling on inflamed endothelium is mediated by P-selectin expressed by adherent platelets (Yang et al. 1999).

Platelet secretion of CD40L was shown to significantly increase phagocytosis by macrophages during infection with *Leishmania amazonensis* (Soong et al. 1996). CD40L also increases reactive oxygen species (ROS) production by neutrophils which enhances their capacity to phagocytose pathogens (Elzey et al. 2011). Platelet activation by LPS via TLR4 induced binding to neutrophils and NET formation leading to entrapment of bacteria (Clark et al. 2007; McDonald et al. 2012). Platelet-neutrophil interaction in NET formation was shown to be LFA-1 dependent in bacterial infection and Mac-1 dependent in viral infection (Jenne et al. 2013b; McDonald et al. 2012). Furthermore, platelet-neutrophil interaction resulting in NET formation was observed not only in the presence of LPS but also in sepsis induced by Gram-negative bacteria (*Escherichia coli*), Gram-positive bacteria (*Staphylococcus aureus*), and viral infections (Jenne et al. 2013b; McDonald et al. 2012). Besides NET formation, LPS-activated platelets induced neutrophil degranulation and increased phagocytosis. Moreover, platelets were demonstrated to be essential for neutrophil responses to pathogen as this was significantly decreased in the presence of TLR2-deficient platelets incapable of pathogen detection (Assinger et al. 2011; Clark et al. 2007). Recently, platelets were identified to be an important surveillance mechanism to resident liver macrophages (Kupffer cells) during blood-borne infection. Trapping of bacteria by Kupffer cells triggers firm adhesion of platelets to their surface, encasing and helping to kill the bacteria (Wong et al. 2013).

Significance of Platelets in Host Defense

Platelets seem to be an active and integral part of the host defense against pathogens as inhibition of platelet aggregation was shown to aggravate acute bacterial infection and worsen outcome (Zhang et al. 2011). Platelets were found to have a protective role during pneumococcal pneumonia independent from their aggregation (van den Boogaard et al. 2015). Moreover, platelet-induced NET formation by neutrophils protects against dissemination of bacterial infection to distant organs (McDonald et al. 2012). Interestingly, some authors have documented that *Streptococcus pyogenes* bacteria can bind to platelets and thus promote dissemination of bacterial infection (Kahn et al. 2013). Therefore, it can be seen that the role of platelets in sepsis is very complex and numerous authors have documented that thrombocytopenia is associated with severely impaired survival in sepsis (Akca et al. 2002; Baughman et al. 1993; Brogly et al. 2007; Claushuis et al. 2016; de Stoppelaar et al. 2014a, b; Moreau et al. 2007; Sharma et al. 2007; Strauss et al. 2002; van den Boogaard et al. 2015; Vanderschueren et al. 2000).

In viral infections, platelets were recently shown to be essential in both host response and survival in infections induced by hepatitis B and C virus (HBV, HCV),

encephalomyocarditis virus (EMCV), lymphocytic choriomeningitis virus (LCMV), oncolytic poxvirus, and myxoma virus (Iannacone et al. 2005; Jenne et al. 2013b; Koupenova et al. 2014; Loria et al. 2013). However, while platelet activation might be necessary for the host defense against certain viruses, it could also promote immunopathology in the affected organ—as shown in viral hepatitis where platelets could mediate cytotoxic T cell-mediated liver damage (Iannacone et al. 2005). Thus, antiplatelet therapy in chronic hepatitis B might potentially reduce chronic inflammation, liver fibrosis, and hepatocellular carcinoma (Aiolfi and Sitia 2015). In addition, in HIV infection, platelets actively capture and transfer HIV-1, possibly facilitating HIV-1 dissemination in infected patients (Chaipan et al. 2006).

Platelets have also been shown to have protective effects against parasitic protozoal infections. It has been noted that there is a strong association between thrombocytopenia and outcome in malaria suggestive of a potential role for platelets in this disease (Cox and McConkey 2010). In this condition, platelets were found to bind infected red blood cells and induce their destruction and intracellular parasite killing via a mechanism involving both PF4 and Duffy antigen (McMorran et al. 2009, 2012, 2013). Platelets were also demonstrated to interact with fungi, and the platelet-derived chemokines, CCL5 (RANTES) and CXCL4 (PF4), exhibit direct antimicrobial activity against *Candida* species (Drago et al. 2013; Netea et al. 2015; Speth et al. 2014). However, the significance of platelets to host defense against fungal infections has to be further investigated. There is an emerging evidence that platelets might be involved in response to *Mycobacterium tuberculosis* infection, specifically by modulation of macrophage function and their transformation into multinucleated giant cells observed in the granulomas characteristic of tuberculosis (Feng et al. 2014; Lugo-Villarino and Neyrolles 2014). However, a distinct role for platelets in the pathogenesis of tuberculosis has yet to be clarified. Lastly, it was speculated that platelets are not only direct effectors of host defense but also function as circulating sentinels that bind infectious agents and present them to the reticuloendothelial system (RES) (Aslam et al. 2006).

Platelets Link Innate Immune Responses to Adaptive Immunity

There is increasing evidence that platelets are involved in adaptive immunity and possibly link innate and adaptive responses together. Platelets have been shown to be involved in T cell responses, to induce B cell isotype switch and dendritic cell maturation (Morrell et al. 2014).

Furthermore, some of the mediators secreted by platelets create a direct link between innate and adaptive immunity. For example, platelet-derived chemokines (PF4, RANTES) and thromboxane were demonstrated to influence T cell trafficking, activation, and differentiation (Morrell et al. 2014; Sakata et al. 2010; Shi et al. 2014; Srivastava et al. 2008; Swaim et al. 2010). Conversely, T cells might activate platelets through CD40L/CD40 interactions, leading to the release of RANTES and increased T cell recruitment (Danese et al. 2004). T_{Reg} differentiation is dependent on TGF-β, and it is therefore of considerable interest that studies of patients with immune thrombocytopenia have demonstrated that platelets might be the primary source of this factor suggesting that platelets might be important for T_{Reg} homeostasis (Stasi et al. 2008; Tran 2012). Platelets were recently shown to enhance T_{Reg} responses by promoting $FoxP3^+$ T cell proliferation and to induce the suppression phase of Th1 responses by TGF-β-mediated selective inhibition of $FoxP3^-$ T cell proliferation (Zhu et al. 2014). Platelet-derived CD40L was shown to significantly promote protective adaptive responses as CD40L augments T cell responses to bacterial and viral challenge and induces maturation of dendritic cells, B cell differentiation, and immunoglobulin isotype switching (Elzey et al. 2003, 2005, 2008, 2011; Sprague et al. 2008). Moreover, platelet CD40L and IL-1β represent major danger signals for dendritic cells, and at least CD40L induces DC maturation.

Together with RANTES, IL-1β and CD40L are also major regulators of DC activity. Platelets were shown to decrease the production of pro-inflammatory cytokines IL-12p70 and TNFα by DCs and to mediate production of anti-inflammatory cytokine IL-10 (Banchereau and Steinman 1998; Gallucci and Matzinger 2001; Kissel et al. 2006). Recently, some authors demonstrated that PDGF inhibited maturation of DCs and induced IL-10 secretion through upregulation of C-type lectin-like receptor 2 (CLEC-2) on DCs and induction of T regulatory cells (Agrawal et al. 2015). Other authors showed that platelets can recruit and activate dendritic cells directly through interaction of Mac-1 on dendritic cells and the platelet junctional adhesion molecule C (JAM-C) (Langer et al. 2007). Lastly, in some bloodstream infections, platelets rapidly associate with a portion of pathogens and subsequently direct the bacteria to dendritic cells inducing antibacterial immunity rather than rapid bacterial clearance (Verschoor et al. 2011). Indeed, platelets were shown to process and present antigen in MHC class I and directly activate naive T cells in a platelet MHC class I-dependent manner (Chapman et al. 2012).

Summary

Platelets, although small and anucleated, are now emerging as powerful entities broadly involved in inflammation, infection, and adaptive immune responses. We have reviewed the role of platelets in different components of the immune

system. Platelets are equipped to sense inflammatory stimuli and to secrete pro-inflammatory, prothrombotic, and proangiogenic mediators as well as being able to directly interact with other immune cells, especially neutrophils and monocytes. Platelets directly interact with bacteria and significantly contribute to bacterial clearance. Platelets create a link not only between hemostasis and immunity but also between innate and adaptive immune responses. Considering the availability of multiple antiplatelet drugs in clinical medicine, it would seem sensible to consider using these agents as potential treatments for immune-mediated diseases.

Take Home Messages

Pathogen Recognition Receptors Expressed by Platelets

- Platelets express several types of PRRs: TLRs 1–9, CD14, CD40, and FPR, allowing them to sense both pathogen- and danger-associated signals.
- Engagement of PRRs on platelets leads to an increase in intracellular calcium, platelet adhesion and aggregation, GPIIbIIIa expression, cytokine release, chemotaxis, and NET formation.

Platelets Secrete Pro-inflammatory Molecules

- Platelets store and secrete over 1000 biologically active proteins, peptides, lipids, and eicosanoids. These include cytokines (IL-1β, IL-8, CD40L, and βTG), chemokines (PF4, RANTES, MIP-1α, and MCP-3), growth factors (PDGF, VEGF, TGF-β), and MHC class I antigen-presenting pathway proteins. Many of these substances contribute to intercellular signaling and inflammatory response at the local and systemic level.
- Some of the stored proteins are constitutively expressed on platelet membrane (GPIIbIIIa, GPVI, PECAM, GPIb-V-IX), while others, such as P-selectin, are exposed on the platelet surface only after platelet activation.
- Soluble platelet mediators are secreted in a paracrine, endocrine, or juxtacrine manner, retained on the platelet membrane bound to the receptor and available for intercellular signaling.
- Platelets contain a wide array of RNAs (mRNA, tRNA, miRNA, long noncoding RNA, circRNA), which role has to be further determined.
- Beside lipid mediators (PAF and TXA_2), platelets could synthesize proteins (IL-1β) in a process called signal-dependent translation.
- Platelet-derived microparticles are an important transport and possibly also storage mechanism for platelet-secreted molecules, like IL-1β, TF, CD40L, RANTES, NFkB, PPARγ, and miRNA.

Platelet Interaction with Immune Cells

- Platelets recruit leukocytes. Adherent platelets promote neutrophil and monocyte rolling on the endothelium via P-selectin and PSGL-1 interaction. The production of chemokines (PAF and RANTES, respectively) allows for firm adhesion of leukocytes. Integrins involved are Mac-1 or LFA-1 on neutrophils and GPIIbIIIa, GPIbα, or ICAM-2 on platelets. In many diseases activated platelets could adhere to leukocytes in circulating blood via P-selectin/PSGL-1 interaction, forming heterotypic aggregates.
- Platelets are recruited by neutrophils. Neutrophils recruited to inflamed vessels extend a PSGL-1-bearing microdomain on the uropod into the vessel lumen and scan for activated platelets expressing P-selectin. Binding of platelets by activated neutrophils induces NET formation. Integrins involved are LFA-1 in bacteria-induced NET formation and Mac-1/GPIIbIIIa in virally induced NET formation. NETs could be formed also in sterile inflammatory processes like deep venous thrombosis.
- Both activated and resting platelets could be recruited by inflamed endothelium. Endothelial ligands P-selectin, PSGL-1, and ICAM-1 recruit platelets via GPIb-V-IX or PSGL-1, P-selectin, and GPIIbIIIa, respectively. Involvement of vWf and GPVI was also suggested.
- Platelet interaction with immune cells is crucial in further propagation and modulation of inflammatory responses.

Platelets in Sterile Inflammation

- The role of platelets in sterile inflammation has been studied in multiple diseases, including atherosclerosis, acute lung injury, ischemia/reperfusion, and autoimmune disorders. Their role was demonstrated mostly as pro-inflammatory, and modulation of platelet activation and their interaction with neutrophils in these diseases often improved outcome.

(continued)

- Platelet-neutrophil interaction or release of IL-1-β-positive microparticles was shown to be in the center of the detrimental pro-inflammatory effects of platelets in sterile inflammation.
- In a localized sterile injury such as occurs following trauma or burns, platelets were shown to be beneficial to the host by augmenting neutrophil recruitment to allow subsequent wound repair.

Platelets in Infection

- Platelets are targeted to the sites of infection and directly interact with microbes. They cluster around microorganisms, encapsulate them, and facilitate their clearance.
- Direct contact of platelets with bacteria, viruses, and parasites may induce phagocytosis of the pathogen by platelets. Microbes reported to be phagocytosed by platelets include *Staphylococcus aureus*, HIV, influenza, dengue, HCV, *Plasmodium vivax*, and *Toxoplasma gondii*.
- Platelets produce several bactericidal and fungicidal proteins and peptides, defensins, thrombocidins (thrombin-induced platelet microbicidal proteins), and kinocidins (chemokines with microbicidal activity) allowing direct pathogen killing.
- Platelets contribute to elimination of pathogens also indirectly by interaction with other immune cells promoting their inflammatory response to pathogens.
- Platelet-neutrophil interaction in bacterial and viral infections results in NET formation and entrapment of the microorganisms. Integrins involved are LFA-1 in bacterial and Mac-1 in viral infection.
- Platelets were identified to be an important surveillance mechanism to resident liver macrophages (Kupffer cells) during blood-borne infection.
- Platelets were found to be indispensable in host defense; however in some infections, like HIV or *Streptococcus pyogenes*, platelets were reported to augment dissemination of infection.

Platelets in Adaptive Immunity

- Platelets have been shown to be involved in T cell responses, to induce B cell isotype switch and dendritic cell maturation.
- Some of the mediators secreted by platelets, such as IL-1β, CD40L, PF4, RANTES, and TXA_2, create a direct link between innate and adaptive immunity.
- Platelets are involved in bidirectional signaling and direct cell interactions with adaptive immune cells.
- Platelets were shown to process and present antigen in MHC class I and directly activate naive T cells in a platelet MHC class I-dependent manner.

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Platelets in Neurological Disorders

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Abstract

Although blood platelets are primarily involved in hemostasis and thrombosis, it is well recognized that they are also implicated in a number of different physiological and pathological events, including inflammation, host defense, and tumor metastasis. In this chapter, we provide an update on the complex interplay between platelets and some complex neurological disorders, such as Alzheimer's disease, depression, Parkinson's disease, and autism. Platelets and neurons share several biological features, and platelets have been regarded as a suitable and easily accessible cellular model to investigate the molecular mechanisms underlying complex neuropathological disorders for many years. Moreover, alterations of platelet function and responsiveness in patients affected by neurological diseases have been widely documented and have fueled the search for platelet-specific tests for diagnostic and prognostic assays. Although this ambitious goal still remains to be pursued, a large number of studies performed on this topic over the last decades have significantly increased our general knowledge on the pathogenesis of complex neurological disorders and have also provided important insights into novel aspects of platelet biology.

Introduction

Platelets are responsible for hemostasis, thrombosis, and wound healing, but recent evidence shows their involvement in many additional physiological and pathological processes, including atherogenesis, chronic inflammation, host defense, tumor metastasis, and neurological disorders. In this chapter, we will discuss the role of platelets in neurological diseases and the possibility to use platelets as a peripheral, easy to access, and reliable biomarkers for the diagnosis for monitoring of neurological disease progression.

The idea of using platelets as peripheral biomarkers for neurological disorders mainly derives from the observation that platelets share many similarities with neural cells and alterations of platelet structure, function, and metabolism are often associated with the onset of some neurodegenerative disorders. A significant amount of work has been performed over the last decades in an attempt to correlate specific aspects of platelet function or dysfunction to selective neurological diseases, in order to define a possible contribution of circulating platelets to these disorders, and to validate platelet-based tests to monitor both the progression of neurological diseases and the efficacy of drug-based therapies. Through all these efforts, the strong relationship between platelets and neurological diseases has been consolidated and the multiple correlations between neuropathologies and platelet dysfunctions have been often characterized at the molecular level. Although in many cases we are still far from having identified specific peripheral biomarkers for neurological disorders, the data collected over the years has strongly increased our general knowledge on the peripheral alterations associated with the onset of complex

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neurological diseases, and also on specific aspects of blood platelet function.

Similarities Between Platelets and Neurons

Platelets and neurons have different embryological origin, as platelets are produced by fragmentation of megakaryocytes, polynucleated precursors of mesodermic derivation (Anastassova-Kristeva 2003), whereas neurons derive from ectoderm (Bjornsson et al. 2015). There are, however, reports erroneously indicating that platelets, as neurons, are of ectodermic origin. This derives for the misleading interpretation of the early finding that platelets express neuron specific enolase (Marangos et al. 1980), which, however, has been subsequently reported to be expressed in almost in all human tissues (Hullin et al. 1980). Nevertheless, although platelets and neurons have different embryological origin, many proteins specifically expressed in the brain are also present, at unusually high concentrations, in platelets. For instance, the neuronal glycoprotein reelin, that regulates cell migration, synaptic plasticity, and neuronal positioning (Assadi et al. 2003), is also expressed in plasma and in blood platelets (Tseng et al. 2010). Plasma reelin probably originates from the liver and is subsequently taken up by platelets and stored into α-granules. However, synthesis of reelin by megakaryocytes and megakaryocytic cell lines has also been documented (Chen et al. 2007). Therefore, platelets may release reelin upon secretion and reelin has been found to interact with platelets to regulate adhesion and spreading and to modulate thrombin generation (Tseng et al. 2010, 2014). Moreover, abnormalities of platelet function have found in neuronal disorders associated with defective reelin signaling (Botella-Lopez et al. 2006).

Platelets also express plasma membrane receptors which are typically expressed in neurons, where they participate in cell–cell contact and networking, including Eph (Murai and Pasquale 2011; Prevost et al. 2004), semaphorins (Rivera et al. 2009), and amyloid precursor protein (Van Nostrand et al. 1990). Importantly, as with neurons, platelets express a specific transporter for serotonin (Rausch et al. 2005), whose action is essential to keep very low serotonin plasma levels and is responsible for its accumulation into platelet dense granules (Yubero-Lahoz et al. 2013).

An important feature that associates platelets and neurons is the secretory nature of both cells types. Biogenesis of granules occurs through similar pathways, and platelet granule secretion is homologous to granules exocytosis in neurons (Reed et al. 2000; Goubau et al. 2013). Platelets contain specific secretory granules that are released upon activation: α-granules, that mainly store peptides and large proteins, and dense granules packed with different small molecules and bioreactive amines. During thrombus formation, the content of platelet granules is released into the bloodstream and into the inter-platelets space to recruit new cells and to reinforce platelet activation by an autocrine/paracrine mechanism involving bioreactive proteins.

Similarly, neurons have granules with different contents that are released into the synaptic space, where different neurons come into proximity. Neurons contain different secretory granules: small synaptic vesicles (containing GABA, glutamate, acetylcholine), small dense core vesicles (containing serotonin, dopamine, ATP), and large dense core vesicles (containing neuropeptides, growth factors, and hormones). The latter ones have been compared to platelet α-granules, that are heterogeneous and contain a wide range of polypeptides, growth factors, chemokines, cytokines, and adhesion molecules. Similarly, platelet dense granule content is very similar to that of small dense core vesicles (Von Bartheld and Altick 2011).

Proteins stored into granules are synthesized and packaged in the body of the cells and, in neurons, they are then transported to the synaptic end along the extended axon. Similarly, platelet granules are generated inside the megakaryocyte where, at the late stage of maturation, move through cytoplasmic extensions to the proplatelets tips, where they are packed into the released platelets. Trafficking and intracellular relocation of granules are driven by microtubule structures both in neurons and megakaryocytes (Battinelli et al. 2007; Hirokawa et al. 2010).

In circulating platelets, molecules stored granules may also be taken up by endocytosis from plasma. This process involves the formation of multivesicular bodies that represent intermediate compartments for storage and sorting. Multivesicular bodies are also present in neurons, where they participate in compartmentalization of endocytosed materials (Von Bartheld and Altick 2011). Mutations that alter normal trafficking of granules have been identified in many diseases known as "inherited disorders of trafficking." Interestingly, these disorders affect both platelets and neurons and are characterized by bleeding problems and neurological dysfunctions (Gissen and Maher 2007).

One of the most relevant common features between neurons and platelets is their ability to store several neurotransmitters and biological amines (5-HT (serotonin), dopamine, GABA, glutamate, and others). In this context, the ability of platelets to efficiently sequester and accumulate large amounts of serotonin has been considered the most relevant neuron-like feature of these cells for a long time. Serotonin is an important modulator of neural activity and plays a critical role in the control of behavior and in the development of several neuropsychological conditions. Moreover, drugs targeting the serotonin system are widely used in the control of mental and neurological disorders, such as depression, obsessive-compulsive disorders, anxiety, and others (Berger et al. 2009).

In spite of its great relevance for the biology of the brain, the largest amount of bodies serotonin localizes outside the

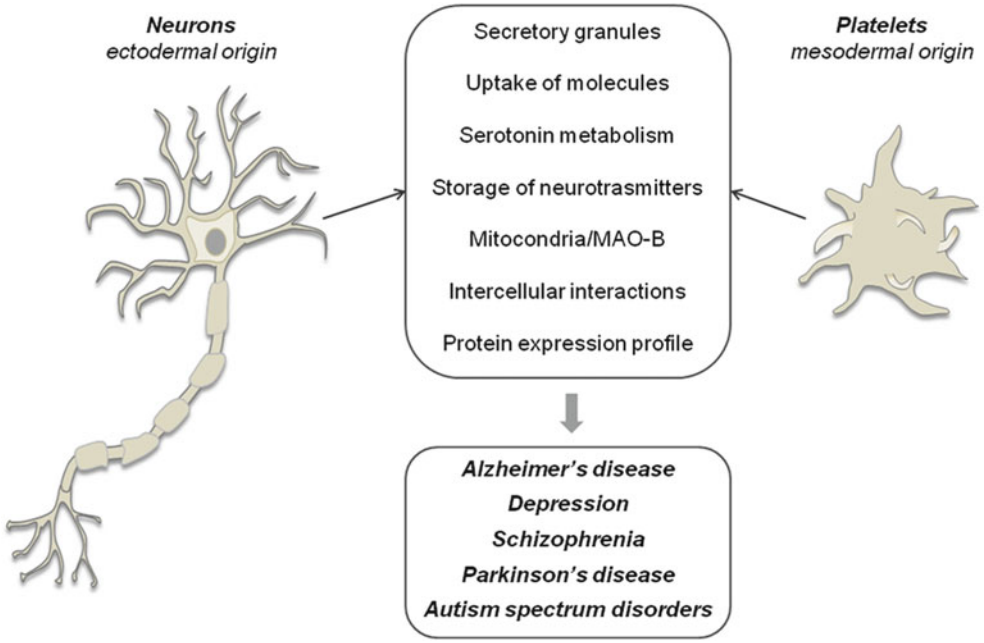

Fig. 1 Similarities between neurons and blood platelets. The major features and characteristics common to both neuronal cells and platelets are summarized. They are discussed in this chapter in relation to the indicated neurological disorders

nervous system, where it supports the activity of different organs and systems. Indeed, serotonin contributes to the control of breathing, cardiac function, as well as the gastrointestinal and genitourinary systems. In addition, serotonin is also a critical player in vascular biology and regulates blood pressure, hemostasis, and platelet function. Within the circulation, platelets themselves store high amounts of serotonin (Berger et al. 2009). Platelets do not express the enzymes required for the synthesis of serotonin, but they are able to take up plasma serotonin through specific membrane transporters and store it into dense granules (Ni and Watts 2006). During platelet activation the content of dense granule is secreted and serotonin acts a weak platelet agonist by a paracrine mechanism, contributing to enhance platelet stimulation and aggregation induced by other agents. These effects are mediated by G protein-coupled 5HT2 receptors that, through the activation of phospholipase C, lead to Ca^{2+} mobilization and platelet activation (Pletscher 1987). Interestingly, inhibition of serotonin reuptake can prolong bleeding time and patients under therapy with selective serotonin reuptake inhibitors display reduced platelet aggregation (Berger et al. 2009; Williams 2012).

Besides the shared physiological features, the possibility to consider platelets as peripheral models for neurons is also supported by the evidence that alterations of platelet function are often associated with neurological disorders and have been found to somehow mirror abnormalities observed in neurons. For instance, mitochondrial dysfunctions are often associated with psychiatric disorders including depression, bipolar disorders, schizophrenia, and anxiety. Defects in mitochondrial function may result in generation of reactive species, alteration of the oxidative phosphorylation and impaired energy production, unbalanced regulation of apoptosis and Ca^{2+} homeostasis, and alteration of monoamine oxidase (MAO) activity (Rezin et al. 2009; Asor and Ben-Shachar 2012). Under some circumstances, these defects are also mirrored by platelet mitochondria, and therefore analysis of mitochondria in platelets has been considered a reliable strategy to gain information on brain mitochondria dysfunctions. In this context, most studies have been performed on platelets isolated from schizophrenic patients, focusing in particular on the function of mitochondrial complex I, cytochrome C oxidase, and MAO-B. Conversely, drugs that target neurons also have an impact on platelets. Several compounds with psychotropic activity, such as the endocannabinoids, 2-arachidonoylglycerol, and anandamide, have been shown to act as agonists for human platelets activation through binding to the endocannabinoid receptors CB1 and CB2 present on circulating platelets (Maccarrone et al. 1999; MacCarrone et al. 2001; De Angelis et al. 2014). Endocannabinoids are also released by platelets and regulate hematopoiesis and atherosclerosis (Jiang et al. 2011; O'Sullivan 2015). Moreover, endocannabinoids also control platelet formation by human megakaryoblasts (Gasperi et al. 2014). Some of the major similarities between platelets and neurons are summarized in Fig. 1.

Implication of Platelets in Complex Neurological Disorders

The possibility to use blood platelets as peripheral biomarkers for complex neurological disorders has been considered a very attractive opportunity for a long time because of the evident technical advantages deriving from analysis of peripheral blood samples rather than brain

biopsies. Therefore, the morphology, structure, and function of platelets in patients affected by different neurological disorders have been intensively investigated since the early 1960s. These studies have provided a significant contribution to our knowledge of the platelet function and the implications for neurobiology, but in most cases this has not yet led to the definitive identification of specific and reliable peripheral biomarkers for diagnostic and prognostic purposes. As a matter of fact, the main interest of the scientific community appeared to switch from pathology to pathology over the years. For instance, the number of available publications clearly indicates that the interest for the involvement of platelets in depression or schizophrenia was maximal in the 1980s and then progressively reduced, despite the number of studies on this topic still remaining significantly elevated compared to other neurological disorders (Fig. 2). In contrast, the studies of the contribution of platelets to Alzheimer's disease have increased slowly, but constantly, since the late 1980s, indicating that this topic is still considered a promising field of investigation. Here we summarize the state of the art of our knowledge concerning the implications of platelets on selected neurological disorders, focusing on those pathologies that have received greatest attention over the years.

Platelets and Alzheimer's Disease

Metabolism of Amyloid Precursor Protein in Blood Platelets

Alzheimer's disease (AD) is the most invalidating dementia in western Countries. It affects over 45 million people worldwide and, together with the aging of the population, it is estimated to grow exponentially in the coming years (World Alzheimer Report 2015). AD is characterized by the abnormal deposition in the brain of senile plaques, composed of amyloid beta (Aβ) peptides derived from the altered metabolism of the amyloid precursor protein (APP). APP is a large glycoprotein expressed on cellular membrane and it is metabolized by two different, mutually exclusive, pathways (Gandy 2005). In the non-amyloidogenic pathway, APP is cleaved by α-secretase, producing soluble APPα and a carboxy-terminal membrane-bound CTFα fragment CTFα is then proteolyzed by γ-secretase complex to release the non-toxic peptide p3. Alternatively, APP may be metabolized by β-secretase, that releases a soluble APPβ fragment, and subsequently by a γ-secretase complex that produces and releases the neurotoxic peptide Aβ40 or the more fibrillogenic peptide Aβ42 (Canobbio et al. 2015).

Although AD is prevalently known as a neurological disorder, it is now well accepted that it actually represents a systemic disease. In fact, deposits of Aβ peptides are also found in cerebral vessel walls, where they may cause cerebral amyloid angiopathy. In addition, amyloid deposits are present in peripheral blood vessel walls. There is solid evidence that the onset of AD is related to alterations of the vascular system and is associated to vascular disorders, including stroke, atherosclerosis and hypertension (Honig et al. 2003; Mielke et al. 2007), and that AD patients suffer often thrombotic and hemorrhagic complications (Brundel et al. 2012). Conversely, several vascular risk factors may increase the risk for AD (Viswanathan and Greenberg 2011).

In the last decade increasing evidence has suggested that circulating blood platelets may be a reliable and accessible model for studying AD pathology and may represent a useful biomarker for diagnosis and prognosis of this disease (Cattabeni et al. 2004; Casoli et al. 2010; Rembach et al.

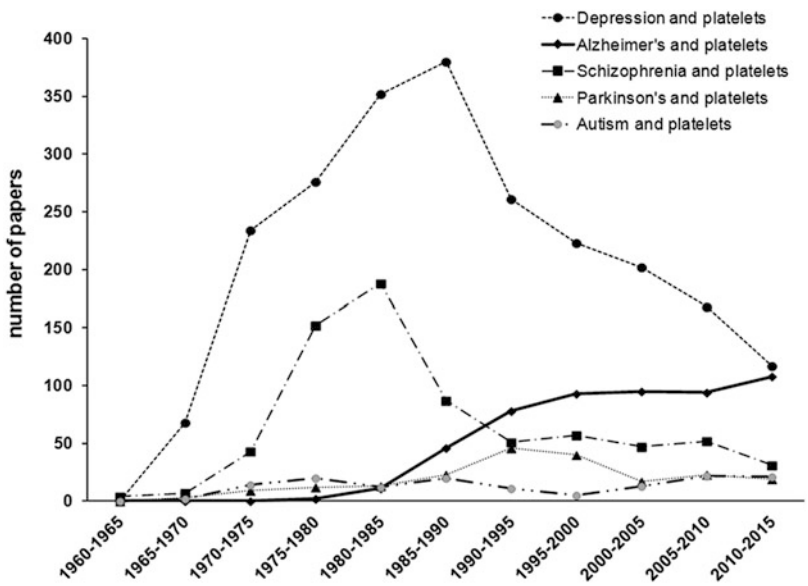

Fig. 2 Platelet studies related to neurological disorders. A search on the PubMed database was performed joining the term platelets and the name of the disorders (i.e., platelets AND autism), including autism, Alzheimer's disease, Parkinson's disease, schizophrenia, and depression. The number of published papers is plotted versus periods of time of 5 years from 1960

2013; Veitinger et al. 2014). Platelets express relatively high amounts of APP (9300 copies per platelet) (Burkhart et al. 2012). APP expressed on plasma membrane may function as a cell–cell receptor (Dawkins and Small 2014; Nalivaeva and Turner 2013) and it is able to bind Aβ peptides (Lorenzo et al. 2000). Platelets contain α-, β-, and γ-secretases necessary for APP metabolism, and process APP through pathways identical to those occurring in neurons (Di Luca et al. 2000). Platelets express two isoforms of APP on the plasma membrane, APP751 and APP770, containing the Kunitz type serine protease inhibitor domain KPI, which is known to inhibit several serine proteases of the coagulation cascade (Van Nostrand et al. 1989). In physiological conditions, platelets metabolize APP mainly through the non-amyloidogenic pathway operated by α- and γ-secretases. It has been shown that α-secretase is activated by platelet stimulation through Ca^{2+}-calmodulin dependent mechanism and that APP hydrolysis can be initiated in vitro by platelet treatment with the calmodulin antagonist W7 (Canobbio et al. 2011). Since non-amyloidogenic and amyloidogenic pathways are mutually exclusive, it is clear that improving non-amyloidogenic metabolism of APP may have significant impact on the onset of AD. In the central nervous system, APP has neurotrophic and neuroprotective actions (Kogel et al. 2012), but little is known on its physiological function in the circulation. Recombinant soluble APP has been demonstrated to inhibit platelet activation in vitro (Henry et al. 1998). APP, as well as the purified Kunitz protease inhibitor domain of APP, inhibits the activity of blood coagulation factors IXa, XIa, and Xa (Smith et al. 1990; Van Nostrand et al. 1992; Schmaier et al. 1993; Mahdi et al. 1995; Wu et al. 2012), indicating that platelet APP may be implicated in the regulation of hemostasis. Recently, Xu and collaborators have demonstrated that transgenic mice displaying a modest overexpression of APP either in platelets or in brain display a marked antithrombotic phenotype. In particular, they demonstrated that a twofold higher expression of APP in platelets caused a marked inhibition of cerebral thrombosis in vivo. In contrast, APP deficient mice showed a significant increase in thrombosis (Xu et al. 2005, 2007). These results indicate that APP actually regulates cerebral thrombosis.

Under pathological conditions platelets may also process APP through β- and γ-secretases (the amyloidogenic pathway) to produce and release Aβ peptides. Platelets are the second source of Aβ peptides after neurons, and the major source of Aβ peptides found in the circulation. Platelets store considerable amount of Aβ peptides in their α-granules (84 ng/g tissue of Aβ40 and 1.6 ng/g tissue of Aβ42) (Kokjohn et al. 2011) and release them in the bloodstream upon stimulation with physiological agonists. In the circulation, platelet-derived Aβ peptides activate platelets (Herczenik et al. 2007; Shen et al. 2008; Sonkar et al. 2014; Canobbio et al. 2014), endothelial cells (Ghiso et al. 2014), and neutrophils (Achilli et al. 2014), resulting in chronic vascular inflammation. Herczenik and coworkers showed that fibrillar Aβ40 induces platelet aggregation by binding to the scavenger receptor CD36 and to GPIbα, and by activating intracellular signaling pathways involving p38MAPK, cyclooxygenase1, and thromboxane A_2 production (Herczenik et al. 2007). Treatment of platelets with Aβ40 also promotes reactive oxygen species production, caspase activation, and membrane scrambling, indicating enhanced platelet apoptosis (Gowert et al. 2014). The ability of Aβ peptides to activate platelets has also been investigated using the synthetic peptide $Aβ_{25-35}$, which represents the biologically active region of Aβ (Kaminsky et al. 2010). $Aβ_{25-35}$ activates platelets, resulting in integrin $α_{IIb}β_3$ inside-out activation and aggregation (Shen et al. 2008; Canobbio et al. 2014). In the presence of extracellular $CaCl_2$, $Aβ_{25-35}$-induced platelet activation is promoted by an increase in intracellular Ca^{2+} concentration, which is likely related to the ability of Aβ peptides to form cations permeable pore on the plasma membrane (Di Scala et al. 2014). Ca^{2+} increases promote granule secretion and release of ADP, which in turn enhances platelet activation (Canobbio et al. 2014). Aβ peptides are also able to induce platelet adhesion under static conditions and to accelerate platelet adhesion to subendothelial matrices under flow (Canobbio et al. 2011). In vivo, preincubation of platelets with Aβ peptides increases platelet adhesion to injured carotid artery. Similarly, in AD transgenic mice, platelets are recruited to vascular amyloid deposits (Gowert et al. 2014).

It has been hypothesized that, by metabolizing membrane APP and releasing Aβ in the bloodstream, platelets may also contribute to the deposition of Aβ peptides in the brain. It is known that Aβ undergoes active transport across the blood–brain barrier (Deane et al. 2003; Deane and Zlokovic 2007). Recently, it has been demonstrated that intraperitoneal inoculation of Aβ-rich extracts induces β-amyloidosis in the brain of AD transgenic mice, although to a lesser extent than intracerebral inoculation (Eisele et al. 2010). This evidence sheds new light on the possible involvement of circulating Aβ in the deposition of amyloid in the brain and the progression of AD, but further investigations are certainly needed.

Another important morphological hallmark of brain neurons in AD is the presence of neurofibrillar tangles, abnormal intracellular deposits of the microtubule-associated protein tau in its hyperphosphorylated form. Maccioni and coworkers have recently shown that platelets also express tau (Neumann et al. 2011). This study investigated tau expression in platelets by immunoblotting and immunofluorescence, and reported also the presence of high molecular weight oligomers of tau, more evident in AD patients compared to healthy subjects (Neumann et al.

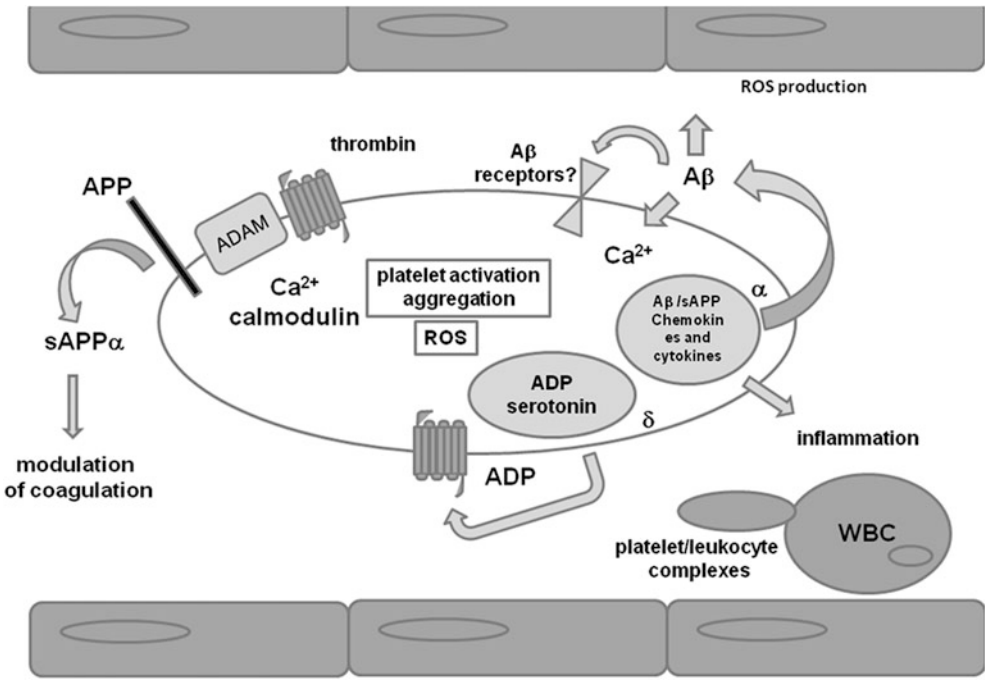

Fig. 3 Amyloid precursor protein metabolism in platelets and implications for Alzheimer's disease. Platelet express amyloid precursor protein (APP) on the plasma membrane and possess α, β, and γ secretases required for its metabolism. APP is proteolyzed through Ca^{2+}-calmodulin activated ADAM and releases soluble APPα that negatively regulates platelet activation. Soluble APP fragments including the amyloidogenic peptide Aβ are also stored in α-granules and released upon platelet stimulation. Secreted Aβ peptides activate platelets possibly by binding to a still unidentified receptors or by directly promoting extracellular Ca^{2+} influx to stimulate and potentiate platelet aggregation. Aβ peptides also activate leukocytes (WBC) and endothelial cells triggering inflammation and favoring platelet–leukocyte interactions

2011). The presence of tau in human platelets has been confirmed by the study of Mukaetova-Ladinska et al. (2012), but it is not supported by proteomic studies that failed to reveal the presence of tau protein in platelets (Burkhart et al. 2012). This discrepancy may be due to the fact that proteomic analysis covers about 80–85 % of the entire proteome and therefore may miss some proteins. However, further analyses are needed to definitively confirm the presence of tau in platelets and its role as a potential peripheral biomarker for AD. It is of interest, however, that glycogen synthase kinase 3β (GSK3β) is the kinase responsible for tau phosphorylation in neurons and that GSK3β activity is increased within the AD brain (Hooper et al. 2008; Balaraman et al. 2006). GSK3β is constitutively expressed in all cells, including platelets (Barry et al. 2003), and is primarily inhibited via Akt-mediated phosphorylation (Cross et al. 1995). Platelet GSK3β has been shown to be upregulated in mild cognitive impairment and AD patients (Forlenza et al. 2011). The complex interplay between platelets and AD is illustrated in Fig. 3.

Platelet Abnormalities in AD Patients

There are several studies documenting that alterations of platelet functionality and metabolism are common in patients with AD (Veitinger et al. 2014). Nevertheless, caution is recommended when considering the data found in the literature, as the methodology and the approach adopted may significantly affect the outcome of the study.

One of the most important and reliable changes observed in platelets from AD patients is the altered ratio among different APP isoforms (Schlossmacher et al. 1992). Immunoblotting analysis of platelet lysates with the anti-APP antibody 22C11, whose epitope is located in the extracellular N-terminal domain of APP, typically reveals two reactive bands with apparent molecular mass of about 130 and 110 kDa, which have been suggested to possibly correspond to the full length APP and to soluble fragments (sAPPα/sAAPβ). The 130 kDa/110 kDa bands ratio is lower in platelets from patients affected by mild cognitive impairment and sporadic AD compared to platelets prepared from either control subjects or from patients affected by other kinds of dementia (Di Luca et al. 2000; Borroni et al. 2005, 2010; Chatterjee et al. 2015). The altered APP ratio may be suggestive of an enhanced metabolism of the protein in platelets from AD patients. Moreover, the altered APP bands ratio in platelets shows a positive and specific correlation with the progression of the disease. This correlation is also present in preclinical stages of AD, suggesting that this can be a useful biomarker for AD.

AD platelets also show alteration in the expression or activation of α- and β-secretases, which are diminished and

increased, respectively (Colciaghi et al. 2004; Tang et al. 2006). This likely results in increased production of Aβ peptides in plasma. However, all the efforts to measure the plasma levels of Aβ have met with little success: many studies have examined plasma Aβ as a biomarker for AD, but findings from these studies have been contradictory. Some studies detected higher levels of Aβ in plasma from AD patients whereas others were unable to confirm this observation (Irizarry 2004; Blennow et al. 2010). To date, based on the currently available measurements, the level of Aβ peptides in plasma appears to fluctuate significantly among individuals, ranging from 16 to 659 pg/ml, both in healthy controls and in AD possibly because these peptides are adsorbed to a variety of plasma proteins as a consequence of their intrinsic hydrophobicity. This observation hampers the possibility to use Aβ peptide concentrations in plasma as a reliable biomarker of AD (Roher et al. 2009).

A number of other alterations of protein expression and function in platelets have been proposed to be associated with the onset and progression of AD, but reported results are often controversial and the overall picture still remains unclear. A decreased activity of phospholipase A_2 has been reported in platelets from AD patients in different studies performed in the same laboratory (Gattaz et al. 1996, 2004, 2014), which have been questioned by others (Krzystanek et al. 2007). MAO-B is responsible for the degradation of neurotransmitters in the nervous system and is also expressed in platelets (Paasonen et al. 1964). Several studies independently documented an increase in MAO-B expression and activity in brain and platelets from AD patients (Bongioanni et al. 1997; Meszaros et al. 1998; Zellner et al. 2012; Cai 2014). Other studies, however, failed to detect any increase of platelet MAO-B activity related to AD (Mann et al. 1981; Konings et al. 1995; Muck-Seler et al. 2009).

It has been suggested that serotonin levels in platelets may be a marker for AD, although results from different laboratories are rather inconsistent. Some studies demonstrated a decreased serotonin uptake by platelets from AD patients (Koren et al. 1993; Inestrosa et al. 1993), whilst at least two other investigators found no differences (Tukiainen et al. 1981; Arora et al. 1991) or opposite results (Kumar et al. 1995). Further analysis is clearly needed to define the precise role of platelet serotonin in AD pathology.

Progression of AD in the brain also affects platelet reactivity. Sevush and collaborators reported a significant increase in circulating platelet aggregates and leukocyte-platelet complexes, as well as an increase of P-selectin exposure in resting platelets from AD patients, demonstrating that more platelets circulate in a pre-activated state in patients with AD compared to healthy controls (Sevush et al. 1998). More recently, Stellos and coworkers demonstrated that integrin $\alpha_{IIb}\beta_3$ is activated on the plasma membrane of resting platelets from AD subjects (Stellos et al. 2010; Laske et al. 2012). Interestingly, in a 1 year follow-up study, platelet activation parameters were found to correlate with the rate of cognitive decline. Importantly, anti-platelet therapy reduced cognitive decline in AD patients (Laske et al. 2010; Sakurai et al. 2013). The pre-activated state of platelets from AD patients is also confirmed by the increased number of circulating coated platelets in AD patients (Prodan et al. 2008, 2011). Coated platelets are a subset of activated platelets characterized by high pro-coagulant activity (Dale 2005). The hyper reactivity of platelets in AD has also been reported in transgenic mouse models of AD (Jarre et al. 2014; Canobbio et al. 2016). Besides platelet hyper activation, abnormal clot formation and degradation has been observed in AD patients accounting for altered coagulation (Cortes-Canteli et al. 2012). This may be related with the ability of Aβ peptides to bind fibrinogen forming a fibrin clot that is more stable and resistant to degradation (Ahn et al. 2010). The prothrombotic state of AD patients may also depend on increased levels of the adhesive protein von Willebrand factor (Mari et al. 1996), increased thrombin generation (Gupta et al. 2005), and increased activity of coagulation factor VII (Mari et al. 1996). As a result, microinfarcts and cerebral emboli are common in AD patients (Purandare and Burns 2009; Brundel et al. 2012).

In conclusion, the available information clearly demonstrates multiple correlations between platelets and AD. In addition to providing several possible diagnostic and prognostic biomarkers of the disease, whose reliability still requires validation, it also emerged that platelets may have an unexpected causative role in the onset and progression of AD, a possibility that opens up new perspectives in the understanding of this neurodegenerative disorder.

Platelets and Schizophrenia

Schizophrenia is a mental disorder characterized by a broad spectrum of symptoms, which display a great heterogeneity among different individuals and, for each patient, also among different periods of time. Emotional, behavioral and cognitive alterations are commonly reported. The correct diagnosis of schizophrenia is often challenging and, in spite of the extensive research performed, the biological mechanisms causing the disease are largely unknown and no reliable biomarkers have been identified. The chance to consider blood platelets as sources of possible peripheral markers for schizophrenia has thus appeared very attractive. Several studies reported an association between schizophrenia and alterations of platelet MAO activity. Platelets express only the MAO-B form, which is also the most abundant isoform in the brain (Youdim 1988). In 1972, an important study by Murphy et al. documented a reduced

MAO-B activity in platelets from schizophrenic patients (Murphy and Wyatt 1972), and this finding generated a strong impulse to the idea that platelet MAO-B could represent a reliable marker for schizophrenia. Subsequent studies on platelet MAO-B expression and activity were more accurately performed by distinguishing different patient subgroups defined in the light of selected parameters, including specific symptoms, genetic influence, and the effect of treatment (Baron et al. 1984; Baron 1985; Duncavage et al. 1982; Marcolin and Davis 1992). A major problem in this respect is that platelet MAO-B activity was found to vary significantly also in the control healthy population with age and gender, and it is also altered in several other psychiatric disorders (Asor and Ben-Shachar 2012). Moreover treatment with neuroleptic drugs was found to alter MAO-B activity in platelets; because of the difficulties of a precise diagnosis and the methodological issues for a standardized evaluation of MAO activity, the results collected over the years, instead of being conclusive, were rather controversial and inconsistent, and did not allow to establish a straightforward link between platelet MAO-B activity and schizophrenia.

As for other neurological disorders, several investigations have been performed to determine a possible link between dysfunctions in platelet serotonin metabolism and schizophrenia. High levels of platelet serotonin have been found in patients affected by chronic schizophrenia and are associated with auditory hallucinations (Stahl et al. 1983; Jackman et al. 1983; Muck-Seler et al. 1991). Interestingly, schizophrenic patients born in winter display the highest levels of platelet serotonin, whereas birth season has no effect on normal controls. Moreover, in schizophrenia patients, but not in healthy subjects, seasonal variations of serotonin levels were observed, with a positive peak in spring (Jakovljevic et al. 1997; Muck-Seler et al. 1999). A single study also showed that low levels of platelet serotonin can be correlated to non-paranoid schizophrenia (Muck-Seler et al. 1991). Conflicting results have been collected about the effects of antipsychotic drugs on platelet serotonin levels and serotonin receptor density. Similarly, the attempts of defining a correlation between schizophrenia and alterations in platelet serotonin uptake have provided controversial results and essentially suggested that this parameter is unlikely to be useful as a marker for the disease (Asor and Ben-Shachar 2012).

In addition to serotonin, the platelet metabolism of other neurotransmitters critical for the development of schizophrenia has been considered as potential peripheral markers for this disease. Different studies approached the problem by analyzing differences in schizophrenic patients in responses to a variety of parameters including platelet dopamine uptake and platelet activation, downstream glutamate, and α2-adrenergic receptors function. However, these studies failed to provide definitive clear results (Sundram et al. 1994; Baier et al. 2009).

The alteration of platelet function in schizophrenia has also been deeply investigated, since patients under antipsychotic therapy present a higher risk of mortality due to cardiovascular and thrombotic diseases (Asor and Ben-Shachar 2012). However, studies on unmedicated patients reported contradictory results, with some reports showing a stronger platelet responsiveness to ADP (Dietrich-Muszalska and Olas 2009; Yao et al. 1994) and lower sensitivity to collagen and serotonin (Dietrich-Muszalska and Olas 2009; McAdams and Leonard 1992), whilst others have shown that platelet responsiveness to these agonists was unaltered (Dinan 1987). It cannot be excluded that a more precise investigation on specific subpopulations of patients may help to clarify the importance of these observations.

Particular attention has been focused on the platelet prostaglandin metabolism, because alterations in the levels of prostaglandins are potentially involved in the development and the evolution of schizophrenia (Horrobin 1977; Feldberg 1976). A poor sensitivity of platelets to prostaglandin E1 was observed in schizophrenic patients (Kanof et al. 1987), associated with a reduced rise in cAMP and impaired inhibition of platelet aggregation (Kaiya et al. 1990; Ofuji et al. 1989). Platelets from schizophrenic patients also display an altered arachidonic acid metabolism and an increased phospholipase A_2 activity that are normalized by antipsychotic treatment (Gattaz et al. 1995). Analysis of intracellular signaling messengers for platelet activation revealed that schizophrenia is associated with a potentiated thrombin-stimulated activation of phospholipase C and increased levels of diacylglycerol, which can be a precursor for arachidonic acid generation, and inositol tris phosphate, that can be responsible for the raise of intracellular Ca^{2+} concentrations (Yao and van Kammen 1996; Ripova et al. 1997). A more recent study documented an alteration of the metabolism of plasmalogens in platelets from schizophrenic patients, characterized by decreased levels of platelet ethanolamine plasmalogens and docosahexaenoic acid, and increased amounts of choline plasmalogens compared to healthy controls (Wood et al. 2015).

A number of additional alterations of platelet markers have been reported to be associated with schizophrenia, including the levels of glutamine synthetase-like protein, the reduced expression of the tyrosine kinase Fyn, the reduced phosphorylation of GSK3β, and the increased activity of platelet mitochondrial complex I (Ben-Shachar et al. 2007; Burbaeva et al. 2006; Ferreira et al. 2015; Hattori et al. 2009; Dietrich-Muszalska and Kwiatkowska 2014). Nevertheless, despite the very high number of studies performed on this topic, there is a general inconsistency among the reported observations and thus the possibility to reliably exploit blood platelets as biomarkers for schizophrenia still remains elusive.

Platelets and Depression

Depression is bidirectionally linked to cardiovascular diseases. The incidence of major depression is dramatically increased in patients that have suffered a myocardial infarction (Forrester et al. 1992; Williams 2012) and, in turn, people affected by major depression display a higher risk to develop cardiovascular events. Depression is also associated with a faster and adverse progression of cardiovascular disease, resulting in an increased morbidity and mortality (Glassman 2007; Frasure-Smith et al. 1993; Thombs et al. 2006; Lesperance et al. 2002; Seligman and Nemeroff 2015). However, the mechanisms linking depression to cardiovascular diseases are still largely unknown, but platelets have been considered to play an important role in the bidirectional connection between these disorders. In particular, platelet dysfunction in depressed patients is thought to represent a major risk factor for the development of cardiovascular diseases.

Several studies have documented a significant platelet hyperreactivity, measured as platelet aggregation, integrin activation, and granule secretion, in depressed patients compared to healthy volunteers (Musselman et al. 1996; Markovitz et al. 2000; Lederbogen et al. 2001; Shimbo et al. 2002; Walsh et al. 2002; Serebruany et al. 2003). Increased platelet activation was observed in response to the major platelet agonists such as thrombin and collagen, and, importantly, also upon treatment with serotonin. It has been suggested that platelet hyper reactivity to thrombin is associated with increased phosphoinositides metabolism and inositol phosphate production. By contrast no differences in platelet aggregation induced by ADP were observed between depressed and non-depressed patients. Although many studies documented the hyper reactivity of platelets in depression, another study reported decreased platelet aggregation in response to collagen and serotonin in depressed subjects compared to controls (McAdams and Leonard 1992), a discrepancy that, however, may be related to methodological approach, as well as to the general study design (Williams 2012; Parakh et al. 2008).

The correlation between depression and platelets function is also supported by the fact that several biological molecules and pathways known to play important roles in depression, including α-adrenergic, adenosine and glutamate receptors, brain-derived neurotrophic factor (BDNF), and the serotonin pathway, are also part of the platelet signaling machinery (Williams 2012).

Serotonin metabolism is critical in depression and represents the preferential target of antidepressant therapy. Platelets store a significant part of the total body's serotonin, which can be secreted upon endothelial injury and platelet activation, and also express both serotonin transporters and receptors. Therefore, platelets represent an ideal peripheral model to obtain valuable information about dysfunctions of the serotonin pathway associated with major depression. In this context, reduced levels of platelet serotonin have been found in depressed patients and inhibitors of serotonin reuptake cause a strongest reduction of platelet serotonin in depressed people than in healthy controls (Maurer-Spurej et al. 2004, 2007; Muck-Seler et al. 2004). The effect of genetic polymorphisms of either serotonin transporters or receptors associated with depression and platelet function has been investigated, but these studies failed to provide straightforward results (Du et al. 2000; Minov et al. 2001; Zhang et al. 1997; Eley et al. 2004). An interesting possible peripheral marker for depression is the density of serotonin receptor, which was found to be strongly increased in depressed patients, whereas serotonin transporter binding and serotonin uptake rate were downregulated (Hrdina et al. 1995; Schins et al. 2003).

The observation that depressed patients display reduced platelet Ca^{2+} movements induced by stimulation of adenosine receptors, and a potentiated Ca^{2+} response upon stimulation of glutamate receptors suggested that dysregulation of these signaling pathways in platelets could be considered as additional peripheral marker for depressive diseases (Berk et al. 2001a, b). BDNF is an important regulator of neuron function and its reduction is considered among the causes for the atrophy of limbic structures observed in depressed patients (Duman and Monteggia 2006). BDNF is also present in blood, and depressed patients display decreased platelet levels, but increased plasma levels of this factor. Interestingly, improvements in the symptoms of the disease were paired to the normalization of BDNF levels (Serra-Millas et al. 2011).

Mitochondrial dysfunction is implicated in different complex neurological pathologies and it has also been suggested to participate in the development of mood disorders. A recent observation describes alterations of mitochondrial respiratory rate in platelets from patients with diagnosis of depressive disorder, suggesting that this alteration could be adopted as a novel biological marker of depression (Hroudova et al. 2013).

An interesting recent proteomic study performed using 2D electrophoresis revealed that a number of platelet signaling proteins can be differently expressed in depressed patients compared to healthy controls. For instance, the levels of protein disulfide-isomerase A3 (PDIA3) and F-actin-capping protein subunit beta (CAPZB) are significantly higher in patients with major depression than in healthy controls. Conversely, other proteins as fibrinogen β chain (FIBB), fibrinogen γ chain (FIBG), retinoic acid receptor beta (RARB), glutathione peroxidase 1 (GPX1), SH3 domain-containing protein 19 (SH319), and T-complex protein 1 subunit beta (TCPB) are expressed at lower levels in patients with major depression (Huang et al. 2014). In

addition to the expression level, also the activation state of some platelet signaling proteins is altered in depression, and thus has been proposed to represent a valuable peripheral marker. For instance, it has been recently shown that AD patients with depression symptoms display significant lower levels of platelet GSK3β phosphorylation, compared to controls and to AD patients without depression. Reduced GSK3 phosphorylation is typically associated with increased activity, suggesting a possible link between dysregulation of this kinase function and the occurrence of depressive symptoms (Platenik et al. 2014). Another research failed to detect alterations in GSK3β expression or phosphorylation in patients suffering bipolar disorders during depression episodes. However, the same investigation showed that lithium therapy promotes GSK3β phosphorylation and inhibition, that inversely correlates with clinical improvements (de Sousa et al. 2015).

Interestingly, major depression is also associated with an increased mean platelet volume that can be normalized by antidepressant therapy (Ataoglu and Canan 2009; Canan et al. 2012). Nevertheless, it should be noted that platelet volume, along with other platelet physical parameters, can be extremely variable also inside a population of healthy volunteers and can be influenced by age, origin, gender, and genetic factors (de Gaetano et al. 2012), implying that powerful verifications, that include a high number of samples, are required before considering this aspect of platelet biology use as peripheral markers of depression.

Platelets and Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder characterized by slowed movements, tremor, and rigidity, and associated with degeneration of dopaminergic neurons in the substantia nigra region of the brain. The therapy for the motor symptoms of PD is based on the use of dopamine and MAO inhibitors. Through the years, platelets have been adopted as a model to study the uptake of dopamine and L-DOPA in PD patients under pharmacological treatment (Goubau et al. 2014; Boullin and O'Brien 1970; Barbeau et al. 1975). Moreover, platelets have also been used to investigate the molecular mechanisms involved in the onset of PD, and many studies have been focused to outline alterations of platelet MAO-B activity and expression in PD patients. Platelet MAO-B may represent a useful tool to monitor the efficacy of MAO-B inhibitors used in the treatment of PD (Goubau et al. 2014). Several works have reported that PD is associated with an increased MAO activity in platelets (Danielczyk et al. 1988; Steventon et al. 1989; Bonuccelli et al. 1990; Bongioanni et al. 1996; Zhou et al. 2001; Husain et al. 2009). However, other investigations recommended caution in interpreting the correlation between the disease and the levels of platelet MAO-B (Zellner et al. 2012; Kuhn et al. 1998).

An important hallmark of the disease is the presence of intracellular inclusions called Lewi bodies, which are enriched in α-synuclein, a neuronal protein playing a critical role in the development of neurodegenerative diseases (El-Agnaf et al. 1998; Saha et al. 2000; Sung et al. 2001; Recasens and Dehay 2014). α-synuclein is also expressed in platelets, but direct investigations do not support the possibility to adopt the analysis of platelet α-synuclein expression and phosphorylation as an appropriate peripheral biomarker of PD (Michell et al. 2005; Shults et al. 2006; Li et al. 2002).

Mitochondrial dysfunction is a typical trait of PD and is not restricted to neuronal cells. Mitochondrial dysfunctions in platelets from PD patients have been extensively investigated, as they could potentially represent a biomarker for diagnosis of the disease. Several authors reported a significant, up to 50 % reduction of complex I activity in the enriched preparations of platelet mitochondria from PD patients (Parker et al. 1989; Benecke et al. 1993; Krige et al. 1992), whereas others failed to detect any significant difference between healthy controls and PD patients (Blake et al. 1997; Mann et al. 1992). It has been suggested that these controversial results could derive from major differences in the methodological approaches, and, specifically, in the different procedure for enzyme extraction (Antony et al. 2015).

An early study showed that platelets from PD patients display an impaired aggregation in response to ADP and epinephrine, but not to collagen (Sharma et al. 1991) and other investigations suggested that PD could be associated with reduced glutamate uptake in platelets (Ferrarese et al. 1999, 2001), reduced expression of platelet α2-adrenoreceptors (Villeneuve et al. 1985), and increased mean platelet volume (Kocer et al. 2013).

Platelets and Autism Spectrum Disorder

Autism spectrum disorder (ASD) includes a variety of disorders of brain development characterized by difficulties in social interaction, verbal/nonverbal communication, and repetitive behaviors (Subramanian et al. 2015). Autism has been associated with developmental alterations in serotonin signaling (Janusonis 2014). Serotonin blood levels are also elevated in ASD patients (Gabriele et al. 2014). A large number of studies have described and confirmed elevated serotonin levels in circulating blood platelets (platelet hyperserotonemia) (Schain and Freedman 1961; Coutinho et al. 2004; Hranilovic et al. 2008; Tordjman et al. 2013; Anderson et al. 2002). The biological bases for the platelet hyperserotonemia, however, remain unknown.

Hyperserotonemia in autism may rely on increased synthesis of serotonin by tryptophan hydroxylase, increased uptake into platelets through the serotonin transporter, decreased release from platelets, and decreased metabolism by MAO-B (Hranilovic et al. 2008). Some data point out to a synergistic effect of genes regulating serotonin synthesis and degradation in dysregulation of the peripheral serotonin homeostasis of autistic patients (Hranilovic et al. 2008). In addition, variants of the serotonin transporter gene (*SLC6A4*) significantly contribute to hyperserotonemia in autism (Coutinho et al. 2004). More recently, defects in genes *NBEA*, *SCAMP5*, and *STXBP6* which are related with sporadic form of autism have been described and result in smaller dense core in granules compared to normal platelets (Castermans et al. 2010).

Conclusions

The possibility to use platelets as peripheral markers of complex neurological disorders represents a very attractive opportunity, as these cells could provide a non-invasive, reliable, and easy-to-obtain alternative cellular model for neurons. The enormous expectation coming from the possibility to identify specific platelet biomarkers for the diagnosis and prognosis of neurological disorders, however, has been largely disappointed. Despite the high number of studies performed on this topic over the last decades, results from different laboratories still remain highly controversial and in some cases contradictory. Nevertheless, the interest in this fled still remains very high, as all the studies so far performed unequivocally demonstrated that several complex neurological disorders have significant impacts on platelet function, that can be associated with an elevated risk of cardiovascular diseases. There is also a general awareness and agreement that neurological disorders are often very heterogeneous diseases. Thus it is likely that more accurate investigations on platelet physiology, focused on well-characterized specific subset of patients, may provide more reproducible results in the future. It should be remarked, however, that this field of investigation has importantly improved our general knowledge on platelet function and dysfunction. In some circumstances, for instance in the case of sporadic Alzheimer's disease, a novel possible contribution of circulating platelets to the onset and progression of the disease has emerged from a number of preliminary studies. All these findings clearly open up new perspectives, not only in the interpretation of the physiopathology of neurological disorders, but also in the characterization of the roles of platelets beyond hemostasis and thrombosis.

Take Home Messages

- Platelets display many structural and functional similarities with neurons
- Alterations of platelet function are often associated with several neurological disorders
- Platelets are good candidate as peripheral biomarkers for complex neurological disorders
- The study of platelet function and metabolism of selected platelet proteins may help to understand the biochemical alterations and pathogenetic mechanisms of some neurological diseases
- Platelets themselves can contribute to the onset and progression of some neurodegenerative disorders such as Alzheimer's disease.

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The Role of Platelets During Development and Reproduction

Shrey Kohli and Berend Isermann

Abstract

Platelets are an essential component of the hemostatic system in non-pregnant and more so in pregnant women. Low platelet counts during pregnancy can be harmful both to the mother and the fetus and can lead to several complications during and after pregnancy. Studies in mice have demonstrated that platelets conduct important functions that ultimately contribute to normal development of the embryo and successful outcome of the pregnancy. While embryonic platelets are dispensable during intrauterine development, they are strictly required during the neonatal period to control hemostasis and closure of the ductus arteriosus. Conversely, maternal platelets—in addition to hemostasis—also regulate placental function, potentially through platelet-released mediators, and studies in mice suggest that increased platelet activation contributes towards the pathogenesis of hypertensive disorders of pregnancy (HDP). Maternal platelet activation has important but poorly defined functions at the feto-maternal vascular bed. This is exemplified by the crucial role of platelets for placental and developmental failure due to defects in the TM-EPCR coagulation system. The relevance of increased platelet activation for HDP is supported by the beneficial effects of inhibition of platelet activation on pregnancy outcome in women with HDP, as shown in large meta-analyses. However, there remains limited and meager mechanistic information on platelet associated pregnancy disorders, which coupled with the challenges of conducting clinical trials in pregnant women is restricting the development of novel therapeutics targeting platelet dependent patho-mechanisms during pregnancy.

Introduction

Changes in the hemostatic system during pregnancy are well established. During pregnancy the mother acquires a slight pro-thrombotic state, which increases the risk of thrombotic events. This potential disadvantage is thought to be outweighed by a decreased risk of hemorrhage and excessive blood loss during delivery. However the physiological relevance of the acquired pregnancy-associated hypercoagulable state remains unknown. Hypercoagulability is associated with an increase of pro-coagulant coagulation factors (e.g., factors I, V, VII, VIII, IX, X), increased platelet activation paralleled by a decreased number of circulating platelets, and increased microparticles (MP) (Fay et al. 1983; Bretelle et al. 2003; Aharon et al. 2004).

Thrombocytopenia During Pregnancy

Gestational thrombocytopenia, defined as a platelet count below 150,000/μl during pregnancy, is a common phenomenon occurring frequently (~8–10 % of pregnancies) in the

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third trimester (Burrows and Kelton 1993a, b; Boehlen et al. 2000; Sainio et al. 2000), although a moderate drop of platelets is not associated with adverse maternal or fetal outcomes. A moderate thrombocytopenia may reflect an increased platelet turnover or a relative dilution due to an increased plasma volume. Inherited defects in platelet activation or number may also be detected for the first time during pregnancy and other potential causes, such as hepatitis C or hematological disorders, need to be considered as well. However, in the context of other pathologies, such as immune thrombocytopenic purpura (ITP), HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count), or heparin-induced thrombocytopenia, a fall of the platelet count indicates a potential harmful pregnancy-associated disease process and the loss of platelet function itself may indeed become harmful.

Immune thrombocytopenia purpura (ITP) is an autoimmune disorder caused by the development of autoantibodies against platelet glycoproteins affecting about 0.2 % of pregnant women (Gernsheimer 2012). Platelets coated with IgG autoantibodies undergo accelerated clearance through Fcγ receptor-mediated phagocytosis by macrophages, preferably in the spleen and liver, causing a rapid platelet clearance from maternal circulation (Beardsley 2002; Cines and Blanchette 2002; Schwartz 2007). Some antiviral antibodies have been shown to cross-react with platelets, increasing the possibility of platelet antigen presentation by MHC class II on phagocytic cells (Wright et al. 1996). As these antibodies may cross the placenta they may also cause thrombocytopenia in the infant. ITP is therefore potentially harmful to both the mother and the fetus. ITP increases the risk of maternal bleeding especially during delivery and about one fifth of pregnant women with ITP develop moderate or severe hemorrhage (Webert et al. 2003). On the embryonic site intracerebral hemorrhage is a rare (less than 1 %), but dreaded complication, but hemorrhage at other anatomical sites is also possible (Burrows and Kelton 1993a, b; Fujimura et al. 2002; Stavrou and McCrae 2009). Of note, the platelet count nadir may occur after delivery and hence careful postnatal monitoring of platelet numbers is advisable. Due to a lack of reliable laboratory tests it can be challenging to differentiate ITP, a potentially harmful disorder, from the benign gestational thrombocytopenia, which accounts for the majority of thrombocytopenias during pregnancy (65–80 %) (Kadir and McLintock 2011; Gernsheimer 2012). Low platelet numbers (e.g., below 80×10^9/L), in particular during the first half of the pregnancy, are considered to occur only in ITP, but not gestational thrombocytopenia. Other causes of thrombocytopenia have to be excluded (Gernsheimer 2012). Management of ITP in pregnancy has been reviewed elsewhere (Kadir and McLintock 2011).

Platelets in Placental Dysfunction

Hypertensive disorders of pregnancy (HDP) such as preeclampsia and HELLP syndrome are a major cause of maternal, fetal, and neonatal morbidity and mortality affecting approximately 5–7 % of pregnancies (Huppertz 2008). These pregnancy-associated diseases are associated with endothelial dysfunction and a low platelet count. One of the key diagnostic criteria of HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count) is a drop of platelet numbers in the peripheral blood (below 100,000/μl) (American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy 2013). An early drop of platelet numbers before the onset of renal changes has been demonstrated, compatible with a causative role of platelet activation (Hutt et al. 1994). Pregnancy in general, and hypertensive disorders of pregnancy in particular, are associated with increased activity of the hemostatic system, but to which extent the activated coagulation system contributes to the drop in platelet numbers is not known. However, hypertensive disorders are likewise associated with an imbalance of prostacyclin (a vasodilator and platelet inhibitor) and thromboxane (a platelet activator and vasoconstrictor) and hence changes in platelet numbers and activation may be simply the consequence of an underlying disorder. Additionally, platelet properties change in the context of hypertensive disorders of pregnancy, as they display an increase in membrane fluidity, cholesterol concentration (independent of plasma levels), and an increased ratio between unsaturated and saturated fatty acids (Coata et al. 1992; Rabini et al. 1995).

Markers of platelet activation such as β-thromboglobulin, thromboxane β2, platelet factor-4, or soluble P-selectin are associated with hypertensive disorders of pregnancy, reflecting increased platelet activation in these disorders (Pekonen et al. 1986; Louden et al. 1991; Jaremo et al. 2000; Harlow et al. 2002). Interestingly, platelet reactivity and the propensity to secrete ATP also increase in HDPs (Morrison et al. 1985; Hayashi et al. 1999). Further by-products of platelet activation are microparticles. Several studies have suggested an association of pro-coagulant microparticles with pregnancy complications and fetal loss (Laude et al. 2001; Bretelle et al. 2003) and in vitro microparticles isolated from women with pregnancy complications cause endothelial dysfunction in isolated myometrial arteries from healthy pregnant women (Vanwijk et al. 2002). Of note, not only platelet derived microparticles, but also microparticles of other cellular origin are associated with impaired pregnancy outcome (Marques et al. 2012). Indeed, syncytiotrophoblast derived MP interact with thrombin activated platelets, and this

interaction increases when using syncytiotrophoblast derived microparticles (MP) from pregnant women with preeclampsia compared to those from women without preeclampsia (Tannetta et al. 2015). The occurrence of microparticles may hence reflect general cell activation and maybe part of a self-propagating disease process. Accordingly, endothelial cell activation is well established in hypertensive pregnancy disorders, which likely contributes to MP formation and itself may cause platelet activation (Taylor et al. 1998; Kim et al. 2004; Powers et al. 2008). Other potential causes of increased platelet activation in hypertensive pregnancy disorders are activation of the renin–angiotensin–aldosterone system, increased levels of cytokines (TNFα, IL1β), or changed prostacyclin and thromboxane synthesis (Nadar and Lip 2004). Collectively, these studies imply a function of platelet activation and potentially microparticles for pregnancy-associated vascular dysfunction and fetal loss. It is conceivable that platelets either directly cause or propagate the disease process. However causality and potential mechanisms remain to be established.

Relevance of Maternal and Embryonic Platelets in Development

Early megakaryocyte like cells can be found as early as day 7.5 post-coitum (p.c.) (Xu et al. 2001). Thus megakaryocyte and potentially platelets are present in the embryo during the establishment of a cardiovascular system and formation of the hemochorial placenta. This raises the question as to whether platelets may be required for the development of the cardiovascular system or the placenta. Qualitative defects with less responsive platelets owing to absence of the Gαq subunit or the thrombin receptors, protease activated receptor-4 (PAR4) or PAR3, have no effect on placentation or pregnancy outcome (Offermanns et al. 1997; Camerer et al. 2004). Unlike in human platelets, in which PAR1 is the pivotal thrombin receptor required for thrombin dependent platelet activation, PAR4 activation by thrombin primarily fulfills this function in rodents, while PAR1 is dispensable. PAR3 is an accessory thrombin receptor on rodent platelets, enhancing PAR4 activation by thrombin, but it is not strictly required (Coughlin 2000). As both PAR3 and PAR4 deficiency does not impede placental or embryonic development, similar to that observed in Gαq deficient mice, qualitative platelet activation defects are entirely compatible with normal embryonic development and placentation in mice.

However, a severe quantitative platelet deficiency owing to the inactivation of the p45 subunit of the NF-E2 (nuclear factor erythroid derived 2) transcription factor, which regulates megakaryopoiesis and is required for pro-platelet formation, does result in a growth retardation of the embryo and placental malperfusion (Shivdasani et al. 1995; Shivdasani and Orkin 1995; Kashif et al. 2011). The absence of such defects in Gαq or PAR4 deficiency already indicates a function of platelets for placentation independent of their activation potential, or a specific function of p45 NF-E2 independent of platelets for placentation. Specific restoration of p45 NF-E2 expression in trophoblast cells by tetraploid aggregation or lentiviral infection of trophectoderm paired with platelet restoration experiments excluded a function of embryonic platelets and established a function of the transcription factor p45 NF-E2 in trophoblast cells for placentation (Kashif et al. 2011). These studies demonstrated for the first time a function of p45 NF-E2 beyond the regulation of megakaryocyte function and platelet formation. In detail, specific reconstitution of p45 NF-E2 in trophectodermal cell lineages is sufficient to restore placental vascularization and embryonic growth despite persistent platelet deficiency (Kashif et al. 2011). Expression of p45 NF-E2 in cells of the trophectodermal cell lineage is required to restrict extensive syncytiotrophoblast formation and permitting normal placental vascularization and embryonic growth (Kashif et al. 2011). p45 NF-E2 modulates Gcm-1 expression by increasing acetylation of JunD and thus its binding to the Gcm-1 promoter (Kashif et al. 2012). These studies clearly establish that p45 NF-E2 has a cell-autonomous function in trophoblast cells, modulating their differentiation, while embryonic platelets are dispensable for placentation and embryonic development. Whether the regulation of megakaryopoiesis and pro-platelet formation by p45 NF-E2 depends on a similar function as in trophoblast differentiation remains unknown.

Akin to the situation in humans, mice lacking platelets display a hemorrhagic diastasis, which in mice is of minor relevance prenatally, but causes frequently postnatal death (Palumbo et al. 2004) (Fig. 1). Of note, fibrinogen deficiency combined with defective platelet function, either quantitative (p45 NF-E2 deficiency) or qualitative (Gαq or PAR4), is compatible with normal placentation and embryonic development in mice, demonstrating that the primary endpoint of hemostasis, the formation of a fibrin-platelet aggregate (Camerer et al. 2004; Palumbo et al. 2004), is not required for intrauterine development. The combined loss of fibrinogen and platelet function results, however, in postnatal hemorrhagic death likely secondary to trauma during vaginal delivery or mild trauma postnatally. The development defects observed in mice with PAR1 deficiency (which is not expressed on murine platelets) or combined PAR1 and PAR2 deficiency must be conveyed independent of platelets and interestingly depends at least in part on proteases not related to coagulation (Camerer et al. 2010).

Platelets do, however, convey an important postnatal developmental function unrelated to the prevention of hemorrhage. Echtler and colleagues established that platelets are crucial for closure of the ductus arteriosus (DA) which is a physiological arterial shunt connecting the pulmonary artery

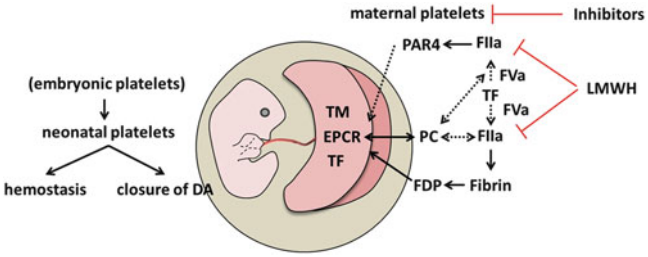

Fig. 1 Scheme summarizing the effects of platelets in reproduction. Embryonic platelets are not required during intrauterine development. However, postnatally they are required for normal hemostasis and they contribute to the proper closure of the ductus arteriosus (DA). Conversely, maternal platelets are required for proper placental function. Through poorly defined mechanisms they contribute to normal placentation. Excess platelet activation via PAR4, e.g. secondary to a disturbed interaction of embryonic (thrombomodulin, TM, endothelial protein C receptor, EPCR, tissue factor, TF) and maternal (thrombin, FIIa; fibrinogen and fibrin, activated factor V, FVa) coagulation regulators at the placental fetal–maternal interface cause placental dysfunction and embryonic demise. These effects can be improved by treatment with low-molecular weight heparin (LMWH) or inhibition of platelet activation

with the aortic arch in the fetus. Its presence in fetal life is required to bypass the non-ventilated lungs. However, its closure within 24–48 h in human newborns is important to avoid cardio-pulmonary complications (Tada and Kishimoto 1990; Hammerman and Kaplan 2001). Patency of DA (PDA) occurs in about 0.2 % preterm neonates and is a major cause of neonatal congenital heart diseases (Mitchell et al. 1971). In this context, platelets play an important role by adhering to DA lumen minutes after birth, which triggers rapid DA closure by thrombotic obstruction (Fig. 1). A quantitative defect of platelets such as in p45 NFE2−/− mice neonates results in DA occlusion defects in about 70 % pups and in persistence of a hemodynamically relevant left-to-right shunt and pulmonary hypertension, leading to right ventricular remodeling (Echtler et al. 2010). These findings are well correlated with human newborns where platelets adhere at the sites of endothelial denudation in constricted DAs but not in PDAs. Additionally, thrombocytopenic preterm neonates had a higher incidence of PDA (68 %) compared to neonates with normal platelet counts (16 %). This indicates an imperative role of platelets in closure of DA in the neonatal period (Echtler et al. 2010).

Unlike the dispensability of embryonic platelets, maternal platelets are required during placentation. Placental tissues from p45 NF-E2 null mothers display large blood pools indicating hemorrhage into the placenta, which, however, does not impair maternal or embryonic survival or embryonic development. This phenotype is specific to maternal platelet deficiency and independent of the embryonic platelet deficiency (Fig. 1). Strikingly, maternal fibrinogen deficiency causes excessive bleeding into the placenta and the uterine cavity, resulting in abortion and hemorrhagic death of the mother in mid-gestation (Suh et al. 1995). These contrasting phenotypes of p45NF-E2 and fibrinogen deficient mice indicate that fibrinogen mediates a crucial function of placentation beyond blood clotting. It is conceivable, but remains to be shown, that fibrinogen, fibrin, or fibrinogen derived peptides have a function during placentation independent of blood clotting, such as tissue-remodeling or cell-adhesion. Intriguingly, low-TF (tissue factor) activity in pregnant female mice results in placental hemorrhage, but also subtle structural alterations within the placenta (Erlich et al. 1999). Whether the impaired TF-activity and loss of fibrinogen share a common mechanism in placentation and whether maternal platelets contribute to these functions remains unknown.

Role of Platelets in TM-EPCR Pathway During Development

While the question as to whether fibrinogen has a function in placentation independent of blood clotting (e.g., the fibrin-platelet aggregate formation) remains unknown, a function of the cell-surface expressed coagulation regulators thrombomodulin (TM) and endothelial protein C receptor (EPCR) independent of blood clotting is established (Healy et al. 1995; Gu et al. 2002). Loss of TM is embryonic lethal at day 8.5 p.c., causing rapid resorption until day 9.5 p.c. Likewise, loss of EPCR causes embryonic death around day 8.5 p.c., but the phenotype is less protruding and resorption of death embryos is protracted (Healy et al. 1995; Gu et al. 2002). The rapid resorption of TM-deficient embryos reflects the dual role of TM both in activating protein C and inhibiting fibrinolysis through activation of thrombin activatable fibrinolysis inhibitor (TAFI, aka as carboxypeptidase N) (Nesheim et al. 1997; Isermann et al. 2003). Increased fibrinolysis and generation of fibrin-split products in TM-null placentae with reduced TAFI activation induces trophoblast cell death and hence rapid resorption (Isermann et al. 2003). The TM-null and EPCR-null embryonic lethal phenotype can be both rescued by trophoblast specific expression of TM or EPCR, respectively, establishing that the expression of both anticoagulant regulators is required on embryonic trophoblast cells at the embryonic maternal interface (Isermann et al. 2001; Li et al. 2005) (Figs. 1 and 2). High levels of thrombin generation in these mice could potentially activate PAR receptors on trophoblast cells of the placenta. Deficiency of PAR1 or PAR2 from the trophoblast and embryo is, however, not protective in TM null embryos, establishing that the placental defect in TM-null embryos is independent of excessive PAR1 or PAR2 activation (Sood et al. 2008). Intriguingly, maternal—but not embryonic—PAR4 deficiency is able to partial rescue TM null embryos (Sood et al. 2008). Additionally, maternal platelet deficiency in p45 NF-E2-null mice or platelet depletion by antiplatelet antibodies likewise partially rescues TM-null embryos

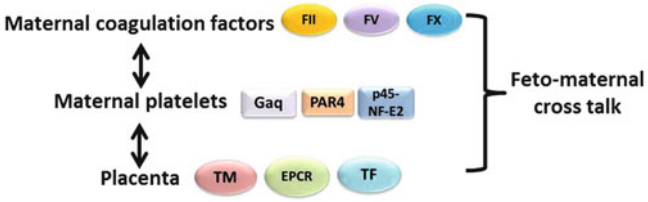

Fig. 2 Maternal coagulation factors and coagulation protease signaling regulated by the TM-EPCR system on trophoblast cells maintain the feto-maternal cross talk at the placental vascular bed and are required for normal placentation, placental function, and embryonic platelets. Maternal platelets play an important mediator in modulating this hemostatic balance and their activation can lead to placental dysfunction and pregnancy failure. *FII* Factor II, *FV* Factor V, *FX* Factor X, *Gαq receptor* Gαq, *PAR4* protease activated receptor 4, *p45-NF-E2* p45 subunit nuclear factor erythroid derived 2, *TM* thrombomodulin, *EPCR* endothelial protein C receptor, *TF* tissue factor

(Sood et al. 2008). These studies establish a role of maternal platelets and platelet activation in regulating the lethality of TM-null embryos (Figs. 1 and 2). The only partial rescue of TM-null embryos in the presence of maternal platelet deficiency indicates the involvement of other unidentified mechanisms or simply partial efficacy in platelet depletion.

The developmental function of TM depends on TF mediated activation of the coagulation system. Expression of tissue factor on the placental trophoblast cells, which are in direct contact with maternal blood, provides a constitutive pro-coagulant stimulus in the placental vascular bed. TM-EPCR-dependent mechanisms, potentially restricting excessive coagulation activation, are required to restrict harmful TF-dependent effects. The precise mechanistic interactions of TM and TF remain unknown. Embryonic lethality of TM-null embryos can be rescued by concomitant embryonic TF-deficiency, establishing a functional interaction. As excessive blood clotting has not been detected in TM-null embryos the death promoting mechanism of TM-null embryos must be independent of occlusive blood clot, but related to a blood-clot-independent interaction between TM and TF. Of note, as platelet deficiency improves survival of TM-null embryos, the mechanism must depend on platelets. Importantly, these findings establish that maternal platelets interact with coagulation regulators expressed within the placenta (TM, TF) and that this interaction of maternal and fetal coagulation components is required for successful placentation. While TF-deficiency rescues the TM-null associated lethality, the reverse is not true. The vascular defect (e.g., in the yolk sac) and embryonic lethality observed in TF-deficient embryos around day 10.5 p.c. persist despite TM-deficiency (Isermann et al. 2003).

The translational relevance of these insights is emphasized by the interaction of a frequent thrombophilic risk factor, the factor V Leiden (FVL) mutation, resulting in resistance to activated protein C, with hypomorphic embryonic TM expressed within the placenta and maternal platelets. The FVL mutation is associated with fetal loss and adverse pregnancy outcomes and the combined presence of maternal FVL and partial loss of TM-dependent protein C activation on trophoblast (by expression of the G404P TM-mutant, TM^{Pro}, with markedly reduced ability to activate protein C) is embryonic lethal (Sood et al. 2007). For example, intercrossing mice with homozygous FVL-mutation along with one TM^{Pro} allele ($TM^{Pro/+}$) shows a complete lethality of $Fv^{qq}TM^{ProPro}$ and fewer $Fv^{qq}TM^{Pro+}$ embryos. TM^{ProPro} embryos in $Fv^{qq}TM^{Pro+}$ mothers possess developmental defects at day 9.5 p.c. and are lost by day 10.5 p.c. The placentae of these embryos are smaller and lack a well-formed labyrinth layer, reflecting a failure of vascular remodeling and potential invasion of embryonic blood vessels into the developing placenta. This placental phenotype is, however, not associated with signs of thrombosis or impaired placental perfusion secondary to thrombosis (Sood et al. 2007). Importantly, maternal platelet depletion starting at day 7.5 p.c., but not at day 9.5 p.c., prevents embryonic loss (Sood et al. 2007). Similar to the results obtained in TM-null embryos, PAR4 deficiency, resulting in platelets unresponsive to thrombin, rescues the developmental block of TM^{ProPro} embryos in $FV^{qqTMPro+}$ mothers. This suggests that increased platelet activation during early placentation (~day 7.5 p.c.) impairs placental development and/or function and proper embryonic development in a pro-thrombotic maternal environment. Intriguingly, the authors consistently were unable to detect blood clots within the placenta. Nevertheless, low-molecular weight heparin (LMWH) treatment, which is expected to dampen coagulation and hence platelet activation, is protective in this model of platelet dependent abortion. Strikingly, while the authors concluded that thrombin mediated maternal platelet activation is central for placental failure, other anticoagulants, including the direct thrombin inhibitor lepirudin, failed to be protective (An et al. 2013). Hence, the precise mechanism causing platelet activation and subsequent placental failure remains unknown.

Role of Platelets on Trophoblast Cells: The Other Side of the Coin

Inappropriate physiological remodeling of maternal spiral arteries is one of the most common phenomena seen in cases of preeclampsia and embryonic growth restriction. Normally, placental cytotrophoblasts differentiate into extra villous trophoblasts (EVTs) that migrate towards the maternal decidua and blood vessels, in particular the spiral artery. Perivascular EVTs disrupt the muscular lining and even replace the endothelium thereby reducing the vascular resistance and ensuring adequate supply of nutrients from the mother to the fetus through the placenta (Ramsey 1981; Khong et al. 1986). Hence a sufficient placental perfusion is

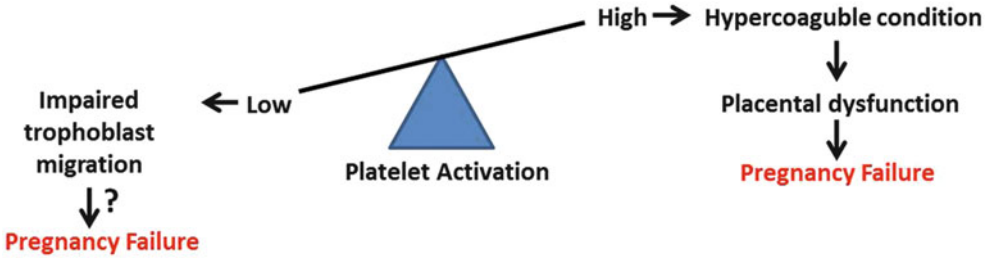

Fig. 3 Model reflecting the requirement of well-balanced maternal platelet activation for successful pregnancy. On one hand, enhanced platelet activation causes impaired placentation and placental dysfunction leading to pregnancy failure and embryonic demise, while on the other hand activation of platelets is required for trophoblast migration and spiral artery remodeling and its failure can likewise promote placental and pregnancy failure

dependent on an efficient migratory system by EVTs. The breaching of maternal blood vessels by EVTs allows direct contact between embryonic trophoblast cells and maternal blood components, including blood cells such as platelets. The direct interaction of trophoblast cells with maternal blood cells is characteristic for the hemochorial placentation as found in humans and mice (Malassine et al. 2003). The relevance of this is exemplified by the interaction of maternal soluble coagulation regulators (e.g., factor V and platelets) and embryonic blood coagulation regulators expressed by trophoblast cells (e.g., thrombomodulin), as described in the previous section. The interaction of maternal platelets with the placenta may, however, extend beyond the regulation of blood clotting. Activated platelets can adhere to isolated EVTs in vitro and—in a matrigel assay—enhance invasion of isolated EVTs via platelet derived chemo-attractants and the receptor CCR1 (Sato et al. 2005). Additionally, the platelets promote a phenotypical switch of ETVs towards an endovascular phenotype, characterized among others by integrin α1 expression (Fig. 3). The exact role of platelets during the process of trophoblast invasion and differentiation and the potential physiological or pathophysiological role of the platelet-trophoblast interaction remains unknown. Of note, the physiological decline of circulating platelets has been attributed to an increased consumption of platelets in the utero-placental circulation, supporting the concept that platelets bind to and accumulate in the placenta (Juan et al. 2011). This assumption remains, however, unproven. Based on the relevance of platelets for embryonic abortion in a hypercoagulable placental environment, one has to assume that excess platelet activation impairs successful placentation, although the underlying mechanism remains poorly defined (Fig. 3).

Clinical Implications and Antiplatelet Therapy

A physiological role of the pregnancy-associated pro-thrombotic state in reducing the bleeding risk at delivery has been proposed. However, at the same time this may increase the risk for adverse outcomes, such as thrombotic events or placental complications. High platelet aggregation and concentration of coagulation factors especially during the third trimester of pregnancy may initiate a vicious cycle of platelet activation, release of platelet derived factors, and further pronounced platelet activation, impairing placental development and pregnancy outcome. As outlined above, only maternal, but not embryonic, platelets are a risk contributor in this setting. However, it is the interaction between the mother and fetus, which is crucial and instigates adverse events at the feto-maternal placental barrier leading to developmental failure and pregnancy loss. Given the contribution of embryonic trophoblast paternal genes and risk factors are expected to contribute to the risk of placental dysfunction—a widely understudied aspect (Galanaud et al. 2010).

Anticoagulants and antiplatelet agents are used to reduce this risk associated with thrombophilia and HDP. In regard to thrombophilia the evidence remains weak at best (Magee et al. 2008, 2014) and careful consideration of the expected benefit and side-effects is required. Anticoagulants such as heparin are associated with a risk of heparin-induced thrombocytopenia or osteoporosis (Nelson-Piercy 1997). Yet, heparins are the most common therapy during thromboembolic events of pregnancy, reflecting the good clinical experience in using these anticoagulants during pregnancy and the fact that they do not cross the placenta (Omri et al. 1989). While anticoagulants (heparins) are established for the treatment of thromboembolic events in pregnancy and have been frequently proposed to convey a therapeutic benefit in thrombophilia associated placental dysfunction, the role of anticoagulants such as heparins during HDP remains unclear.

Considering the above-described role of platelets during placentation antiplatelet agents may be an interesting alternative for HDP. According to a Cochrane review that looked at 39 trials involving >30,000 women, treatment with antiplatelet agents significantly reduces the risk of pre-eclampsia by 15 % (Knight et al. 2000; Duley et al. 2001). Intriguingly, the risk of hypertension in pregnancy was not affected, implying a specific role of platelets in preeclampsia (Knight et al. 2000; Duley et al. 2001). To prevent one case of preeclampsia 85 women need to be treated. Use of

antiplatelet agents was also associated with a small, but significant, reduction in the risk of delivery before 37 weeks (−8 %) or infant death (−14 %). In regard to the risk reduction of HDP, aspirin, the most commonly used antiplatelet agent, showed a beneficial effect. Reliable suggestions for the best dosage regimen cannot be made and the suggestion to use 75 mg or less simply reflects the fact that most studies used such low dosages of aspirin (Knight et al. 2000; Duley et al. 2001). Other platelet inhibitors, such as TXA2 synthase inhibitors, TXA2 receptor antagonists, 5-hydroxytryptamine receptor type 2 blockers, have been proposed, but experience in women with HPD is limited. Clopidogrel has been found to be effective with animal models with no fetal toxicity, but data from human patients is limited (Santiago-Diaz et al. 2009; De Santis et al. 2011). Prasugrel and Ticagrelor are two other recent antiplatelet agents that have been investigated, but to date only in animal studies (Patti et al. 2014). Anticoagulants such as hirudin have been likewise considered, but at face-value heparins, which do not cross the placenta, appear to be a safer alternative (Omri et al. 1989). Endothelin antagonists target specific endothelial dysfunction in HPD and have also shown protective effects in animal models (Olson et al. 1999). By increasing endothelial nitrite oxide endothelin antagonists would also restrict platelet activation. Evaluating the efficacy of antiplatelet therapies for pregnancy-associated complications such as HDP has been limited hitherto reflecting the difficulties to conduct randomized controlled trials in pregnant women. It remains a major challenge to test agents which might be potential relevance and benefit for affected pregnant women.

Take Home Messages

Thrombocytopenia During Pregnancy

- Platelets are essential to maintain the hemostatic balance during pregnancy.
- Gestational thrombocytopenia is characterized by a moderate drop of platelets. It is frequent, but harmless.
- ITP can cause thrombocytopenia in the mother and the infant and requires careful work-up and close monitoring.

Relevance of Maternal and Embryonic Platelets in Development

- Qualitative or quantitative embryonic platelet defects are compatible with normal placentation and embryonic development.
- Postnatally platelets are required for proper closure of the ductus arteriosus and for hemostasis.
- Maternal platelets and fibrinogen are required for proper placentation.
- The transcription factor p45 NF-E2 is expressed in hematopoietic and trophoblast cells. p45 NF-E2 regulates placentation and embryonic development independent of platelets.

Role of Platelets in TM-EPCR Pathway During Development

- Maternal platelets interact with embryonic coagulation regulators expressed within the placenta and regulate the hemostatic balance at the feto-maternal interface.
- Excess maternal platelet activation contributes to the poor placentation and embryonic growth restriction or lethality caused by defects in the TM-PC system.

Clinical Implications and Antiplatelet Therapy

- Antiplatelet agents can reduce the risk associated with thrombophilia and HDP and may constitute an interesting therapeutic approach.
- Further research is required to define the clinical value of antiplatelet therapy for HDP.

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Platelet-Derived Inhibitors of Platelet Activation

A.J. Unsworth, A.P. Bye, and J.M. Gibbins

Abstract

Negative regulators of platelet activation are a relatively unexplored aspect of platelet physiology yet have an important role in tempering thrombus development by contributing much needed negative regulation to a process that is amplified by several positive feedback mechanisms. Some negative regulators, such as RASA3 and JAM-A, act as gatekeepers that modulate key mediators of activation and provide barriers that must be deactivated to permit full activation and stable thrombus formation. Other negative regulators, such as PECAM-1 and other proteins that signal through ITIMs, come into play once platelets are activated and provide restraining, negative feedback for activatory pathways. Many platelet-derived inhibitors have been identified but not fully characterised and so questions remain regarding the mechanisms that underlie the effects on platelet activity following their activation, inhibition or genetic disruption. However, dysregulation of inhibitory signals is believed to contribute to enhanced risk of thrombosis in diseases such as diabetes and other pathological conditions. In this chapter we have described platelet-derived inhibitors of platelet function that are secreted by or expressed within platelets themselves to provide inhibition or negative regulation to the processes that underpin activation.

Introduction

When blood vessels are damaged, circulating platelets come into contact with activating stimuli that trigger aggregation and enable them to form a hemostatic plug. This process is subject to both positive and negative feedback to ensure that platelets respond appropriately to damage and do not form thrombi that totally occlude the vessel. It is believed that dysregulation of negative feedback mechanisms contributes to increased risk of thrombosis associated with some diseases. Despite this association with thrombosis, platelet-derived negative regulators of platelet activation are relatively poorly understood in comparison to mediators of platelet activation. However, it is becoming increasingly apparent that the mechanisms by which platelets restrain activation are diverse and of equal complexity to those that mediate positive signaling. Some regulators, such as RASA3 and JAM-A, act as gatekeepers that must be deactivated before platelet activation can occur. In contrast, regulators that contain ITIMs, such as PECAM-1, are activated following stimulation and mediate negative regulation via phosphatases that restrain activation. Wnt3a and ESAM are thought to directly limit platelet-platelet adhesion by blocking activation of the fibrinogen receptor, integrin $\alpha_{IIB}\beta_3$. The various isoforms of PKC expressed by platelets elicit a diverse and complex array of inhibitory effects including receptor desensitisation. Many platelet derived inhibitors have been identified but not yet fully characterized and so questions remain regarding the details of their roles in the regulation of platelet activity. In this chapter we have described platelet-derived inhibitors of platelet function that are secreted by or expressed within platelets themselves to

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Table 1 Platelet-derived inhibitors of platelet activation

Immunoreceptor tyrosine-based inhibitory motif containing receptors (ITIMs)
PECAM-1
CEACAM1/2
G6b-B
PIRB/LILRB
Intracellular nuclear receptors
PPARs (PPARα, PPARβ/δ and PPARγ)
LXR
RXR
GR
Negative regulators of small GTPases and integrin $\alpha_{IIb}\beta_3$
RASA3
JAM-A
ESAM
Wnt3a
Neuropilin-1 plexin A complex
Cyclic nucleotide signaling
cAMP and PKA
Platelet-derived NO and PKG
Other mechanisms
Phosphatases
Receptor desensitisation
Protein kinase C isoforms (*PKCδ and PKCθ*)

Platelet-derived inhibitors of platelet function that are secreted or expressed by within platelets that provide inhibition or negative regulation to the processes that underpin activation

provide inhibition or negative regulation to the processes that underpin activation (Table 1).

ITIM Signaling

Immunoreceptor tyrosine-based inhibitory motif (ITIM) containing receptors are capable, following ligand binding, of triggering cell signaling mechanisms that counteract activation processes. The ITIM consensus sequence L/I/V/S-x-Y-x-x-L/V usually found in the cytoplasmic tail has been identified in several proteins that are expressed in platelets, including PECAM-1, CEACAM, G6b-B, and LILRB2/PIRB which are associated with the negative regulation of platelet activation (Wu and Lian 1997; Cicmil et al. 2000; Jones et al. 2001; Wong et al. 2009; Alshahrani et al. 2014; Yip et al. 2015; Newland et al. 2007; Mori et al. 2008; Coxon et al. 2012). When ITIM-bearing receptors bind their ligand, the receptors cluster and src family kinases phosphorylate the tyrosine residues in the ITIM motif. The phosphorylated ITIM is then able to recruit negative regulators including phosphatases such as SHP1/SHP2 (Kharitonenkov et al. 1997) and SHIP1/SHIP2 (Bruhns et al. 2000). The recruited phosphatases are localised within close proximity to their substrates, which allows them to inactivate molecules involved in activatory cell signaling including tyrosine kinases and phosphatidylinositol 3-kinase (PI3K) (Fig. 1). ITIMs were initially considered to be the 'off switch' that counteracts the positive signaling initiated by ITAM (Immunoreceptor tyrosine-based activation motif, consensus sequence $Yxx(L/I)x_{6-12}Yxx(L/I)$) containing receptors such as the GPVI (glycoprotein VI) receptor complex. However, studies have now identified negative regulation of GPCR signaling by PECAM-1 and G6b-B that are independent of ITAM signaling pathways (Newland et al. 2007; Jones et al. 2009) (Fig. 1).

PECAM-1

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130 kDa member of the immunoglobulin superfamily that is expressed on several haematopoietic cells including platelets, monocytes, neutrophils, some types of T cells and endothelial cells and is associated with several biological processes including negative regulation of platelet activation (Newman 1997). PECAM-1 contains a 574 amino acid extracellular domain that is composed of 6 Ig-like domains and mediates homophilic interactions with other PECAM-1 molecules. It also contains a short 19 amino acid single transmembrane spanning domain and a 118 amino acid cytoplasmic domain that includes the ITIM (Sun et al. 1996; Newton et al. 1997; Goyert et al. 1986; Lyons et al. 1988). Expression levels of PECAM-1 are variable with copy numbers ranging between 5,000 and 20,000 present at the cell surface per platelet (Jones et al. 2009). PECAM-1 is present in platelet α-granules, resulting in an increase in cell surface expression following platelet activation and granule secretion (Hidari et al. 1997; Wu and Lian 1997; Metzelaar et al. 1991; Jones et al. 2009). PECAM-1 is believed to be activated by homomeric clustering (Thai le et al. 2003) but interactions with other receptors have also been reported (Buckley et al. 1996). Clustering of PECAM-1 to ITAM containing receptors or ligation of PECAM-1 using anti-PECAM-1 antibodies or recombinant human PECAM-1 immunoglobulin chimeras has been shown to inhibit GPVI, GPIb and GPCR-stimulated platelet aggregation. Activation of PECAM-1 has broad effects on signal transduction, including reduced total tyrosine phosphorylation, inositol tris-phosphate production, Ca^{2+} mobilisation and granule secretion which underpins a reduction in thrombus formation (Cicmil et al. 2002; Jones et al. 2001, 2009; Thai et al. 2003; Rathore et al. 2003). PECAM-$1^{-/-}$ platelets exhibit hyperreactivity when stimulated with low concentrations of collagen and CRP (collagen related peptide) but not thrombin, ADP or PAR receptor agonists. Studies that have measured laser-induced thrombus formation in cremaster muscle arterioles in vivo using mice deficient in PECAM-1

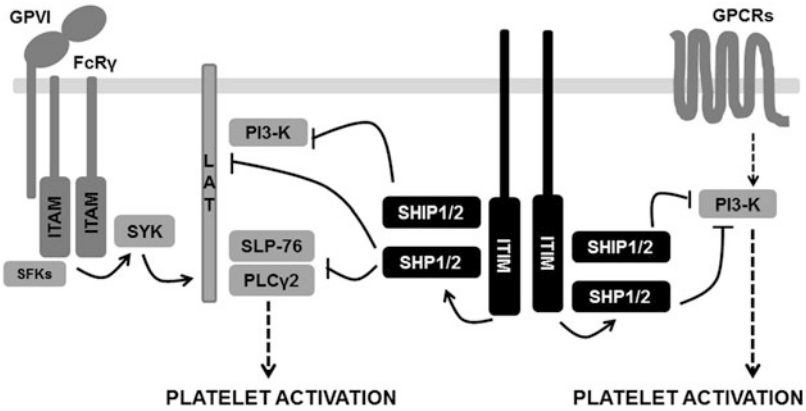

Fig. 1 ITIM signaling. When ITIM-bearing receptors bind their ligand they cluster and the ITIM domains are phosphorylated. The phosphorylated ITIM domains recruit phosphatases through their SH2 domains, bringing them into close proximity with their substrates. The phosphatases SHP1/2 and SHIP1/2 dephosphorylate PI3K, LAT and PLCγ2 and other molecules in the signaling pathways evoked by ITAM containing/linked receptors, such as GPVI, causing inactivation and contributing to negative regulation. The phosphatases recruited by ITIMs are also able to negatively regulate PI3K downstream of GPCRs, contributing negative regulation to non-ITAM signaling pathways

indicate an inhibitory role for PECAM-1 in the regulation of thrombus formation, as increased thrombus size and stability is observed (Falati et al. 2006).

PECAM-1 is regulated by phosphorylation of its cytoplasmic tail at the tyrosine residues Y663 and Y686 within the ITIM and ITSM (immunoreceptor tyrosine-based switch motif) motifs. PECAM-1 is constitutively phosphorylated at low levels in resting platelets and phosphorylation is increased following anti-PECAM-1 antibody induced crosslinking, but also in response to several platelet agonists including collagen, convulxin, thrombin and GPIb agonists suggesting that PECAM-1 provides a negative feedback mechanism to control the level of platelet activation and thrombus formation (Jackson et al. 1997; Modderman et al. 1994; Cicmil et al. 2000; Jones et al. 2001; Rathore et al. 2003). Phosphorylation of PECAM-1 at an ITIM is mediated by activation of Src family kinases and Fyn, Lyn, Src, Yes and Hck have all been shown to coimmunoprecipitate with PECAM-1 (Cao et al. 1998; Cicmil et al. 2000). Phosphorylated ITIMs provide a binding and activation platform for SH2 domain containing proteins, including phosphatases SHP1 and SHP2, SHIP1 and PP2A (Pumphrey et al. 1999; Relou et al. 2003; Henshall et al. 2001) that are associated with the negative regulation of platelet activity. Following activation of GPVI signaling, PI3K associates with the LAT signalosome which is located in lipid rafts that are enriched with the phosphoinositide substrates of PI3K. In contrast to LAT, the majority of PECAM-1 molecules are excluded from lipid rafts. Following crosslinking of PECAM-1, SHP2 is recruited to PECAM-1 and associates with PI3K, relocalising PI3K away from lipid rafts and the LAT signalosome, preventing association with and activation of PI3K (Moraes et al. 2010a). Most recently the mechanism by which PECAM-1 negatively regulates responses to non-GPVI agonists such as thrombin and VWF has also been described (Jones et al. 2014). PECAM-1 inhibits fibrinogen binding and secretion stimulated by thrombin but not PAR1 and PAR4 activating peptides, which suggests that PECAM-1 has a role in regulating GPIbα, a receptor that recruits thrombin to the platelet plasma membrane facilitating stimulation of PARs (Jones et al. 2012). PECAM-1 was found to mediate the internalisation of GPIbα in platelets through dual AKT/glycogen synthase kinase-3/dynamin-dependent and $\alpha_{IIb}\beta_3$-dependent mechanisms.

A study looking at the expression patterns of PECAM-1 in platelets has identified that expression levels of PECAM-1 are variable within the human population with approximately 5,000–20,000 copies estimated to be present at the cell surface. Analysis of the relationship between receptor expression levels and platelet responsiveness to platelet agonists revealed an inverse relationship between levels of PECAM-1 expression and platelet response to stimulation by CRP and ADP (Jones et al. 2009). Although the association is described as modest accounting for 6–10 % of total variability in responses, this was at a similar level of magnitude to that observed for the positive correlation of GPVI or $\alpha_{IIb}\beta_3$ expression levels with platelet responsiveness.

Despite the overall negative role for PECAM-1 in the regulation of platelet activity, PECAM-1 signaling in other cell types is associated with the regulation of integrin function, whereby crosslinking of PECAM-1 enhances adhesion mediated by integrins (Tanaka et al. 1992; Piali et al. 1993; Leavesley et al. 1994; Berman et al. 1996; Varon et al. 1998; Chiba et al. 1999; Zhao and Newman 2001). Studies in human platelets have shown that antibody crosslinking of PECAM-1 enhances adhesion and spreading on fibrinogen (Zhao and Newman 2001) indicating a positive role for PECAM-1 in the regulation of integrin $\alpha_{IIb}\beta_3$. Mouse platelets deficient in PECAM-1 show impaired spreading and adhesion on fibrinogen, clot retraction and phosphorylation of focal adhesion

kinase, suggesting a defect in integrin $\alpha_{IIb}\beta_3$ outside-in signaling (Wee and Jackson 2005). It has been hypothesised that a dual role for PECAM-1 could therefore exist, in which it initially functions to suppress platelet activation but once platelets are strongly activated, PECAM-1 positively regulates functions mediated by integrin outside-in signaling (Jones et al. 2012).

High plasma cholesterol levels significantly increase an individual's risk of atherosclerosis, coronary heart disease, heart attacks and stroke. Statins are widely prescribed as cholesterol lowering drugs and have been shown to reduce platelet activation. It has been described that one possible mechanism by which statins inhibit platelet function is through the activation and regulation of PECAM-1 (Moraes et al. 2013). Treatment of platelets with simvastatin results in increased PECAM-1 phosphorylation and recruitment of SHP2 to the ITIM which are essential for the negative function of PECAM-1. In further support of statins working through PECAM-1, PECAM-1 deficient mouse platelets showed reduced sensitivity to statins compared to WT controls indicating that statins exert their effects via PECAM-1.

CEACAM1 and CEACAM2

Carcinoembryonic antigen (CEA)-related cell adhesion molecules, CEACAM1 and CEACAM2 are ITIM containing membrane receptors that are expressed in both human and mouse platelets. CEACAM1 and 2 both contain extracellular glycosylated Ig-domains (four in CEACAM1 and two in CEACAM2), a transmembrane domain and a long cytoplasmic tail that contains the ITIM which is almost identical between the two proteins (Salaheldeen et al. 2012). CEACAM1 is activated following clustering via a homophilic interaction. The endogenous ligand of CEACAM2 has not yet been identified but can be activated by the murine coronavirus mouse hepatitis virus spike glycoprotein(s) (Robitaille et al. 1999). Studies using other cell types including T-cells and epithelial cells (Nagaishi et al. 2006) have shown that CEACAM1 and CEACAM2 use their ITIMs to recruit SHP1 and, to a lesser degree, SHP2 which can then initiate negative regulation of positive signaling pathways (Beauchemin et al. 1997).

Mice deficient in either CEACAM1 or CEACAM-2 show increased adhesion to fibrillar collagen and increased aggregation and secretion evoked by GPVI which indicates a role for both receptors in the negative regulation of GPVI signaling and platelet responses (Wong et al. 2009; Alshahrani et al. 2014). CEACAM2 deficient platelets have also been shown to exhibit increased platelet responses to CLEC-2 agonist Rhodocytin. Platelets deficient in either CEACAM1 or CEACAM2 show increased tyrosine phosphorylation of Syk and PLCγ2 following stimulation by CRP and also Rhodocytin in CEACAM2$^{-/-}$ platelets. Platelets from mice deficient in either CEACAM1 or CEACAM2 display increased thrombus growth in vitro and in vivo suggesting that CEACAM1 and 2, like PECAM-1, are negative regulators of platelet GPVI signaling. Also similar to that observed with PECAM-1, it has been recently described that CEACAM1$^{-/-}$ platelets show reduced signaling and activation through $\alpha_{IIb}\beta_3$ suggesting an alternative, positive regulatory mechanism for CEACAM1 in platelets (Yip et al. 2015). The role of CEACAM2 in the regulation of integrin $\alpha_{IIb}\beta_3$ is as yet unknown .

G6b-B

The transmembrane protein G6b was identified through both proteomics and gene expression studies (Macaulay et al. 2007; Senis et al. 2007) and the G6b-B variant was confirmed to be present in platelets (Senis et al. 2007). G6b-B contains an extracellular domain consisting of 125 amino acids and a cytoplasmic tail that contains two ITIM sequences. The endogenous ligand of G6b-B has not yet been identified, but G6b-B has been shown to be constitutively phosphorylated in resting platelets, and this increases following stimulation with GPVI specific agonist CRP or thrombin (Senis et al. 2007). Treatment of cells expressing G6b-B with pervanadate to inhibit phosphatases enhances tyrosine phosphorylation of G6b-B and the recruitment of SHP1 and SHP2 (de Vet et al. 2001; Coxon et al. 2012), thereby suggesting that G6b-B works via a similar mechanism to other ITIM containing proteins to inhibit platelets. Interestingly, studies using the DT40 cell line show that inhibition of GPVI signaling following G6b-B expression is retained in the absence of both SHP1 and SHP2, and is also retained in the absence of SHIP suggesting redundancy between these phosphatases or the involvement of other inhibitory molecules and mechanisms of action (Mori et al. 2008). Recent studies have identified that G6b-B is capable of interacting with several key signaling molecules, including Csk, Src, Fyn, Syk, PLCγ2 and PI3K, and it has been suggested that G6b-B may mediate its inhibitory effects on signaling and platelet activity by redistributing signaling molecules away from their substrates (Coxon et al. 2012). Further evidence of a negative regulatory role for G6b-B in platelets was found by using a crosslinking antibody for G6b-B which caused inhibition of platelet aggregation to CRP, and ADP. No alteration in ADP-stimulated Ca^{2+} signaling was observed suggesting that G6b-B acts downstream of Ca^{2+} release. This suggests that G6b-B may act via an alternative inhibitory mechanism to that observed downstream of other ITIM containing receptors such as PECAM and CEACAM, where G6b-B is capable of inhibiting signaling events downstream of mobilisation of intracellular Ca^{2+} (Newland et al. 2007). However, G6b-B deficient mice do not show platelet hyperreactivity, although this is likely to be attributed to an

increase in GPVI receptor shedding, that is observed as a result of enhanced metalloproteinase production in the megakaryocytes of these mice (Mazharian et al. 2012). This indicates an important role for G6b-B in megakaryocytes but the physiological role of G6b-B in platelets remains unclear (Mazharian et al. 2012).

PIRB/LILRB

The leukocyte immunoglobulin like receptors (LILRs) include two subfamilies, LILRA and LILRB, whilst the LILRA proteins contain an ITAM domain, the LILRB family members are characterised as containing an ITIM. Human platelets express LILRB2 and mouse platelets express its homolog PIRB which contains four cytoplasmic ITIMs (Takai 2005). Platelets have also been found to express the PIRB ligand ANGPTL2, which is found in alpha granules, and may suggest that autocrine self-negative regulation of platelets via PIRB/LILRB2 following their activation may occur (Zheng et al. 2012; Fan et al. 2014). Recent studies have identified that treatment of platelets with purified ANGPTL2 results in an inhibition of their activation to several agonists including CRP, ADP and thrombin while adhesion and spreading on fibrinogen is also inhibited (Fan et al. 2014). PIRB-TM mutant mice, which are unable to mediate intracellular signaling through this receptor, have a hyper-reactive platelet phenotype with increased aggregation evoked by CRP, increased spreading on fibrinogen and increased clot retraction. Key GPVI signaling events following activation by CRP including phosphorylation of LAT, SLP-76 and PLCγ2 are inhibited following ANGPTL2 treatment and increased in PIRB-TM mutants. During adhesion to fibrinogen, phosphorylation of FAK and β3 is also enhanced in PIRB-TM mouse platelets. PIRB dependent inhibition of GPVI and integrin $\alpha_{IIb}\beta_3$ signaling has been linked to the recruitment of SHP1 and SHP2 phosphatases, as both are recruited to PIRB and are phosphorylated following treatment with ANGPTL2 but their recruitment and phosphorylation is reduced in PIRB-TM expressing mice (Fan et al. 2014).

Intracellular Nuclear Receptors

Several intracellular nuclear receptors have been identified and characterised in human platelets including the peroxisome proliferator activating receptors (PPAR)s, PPARα, PPARβ/δ and PPARγ, the retinoid X receptor (RXR), liver X receptor (LXR), farnesoid X receptor (FXR) and glucocorticoid receptor (GR) (Moraes et al. 2005a, 2007, 2010c; Spyridon et al. 2011; Ali et al. 2006a) (Fig. 2).

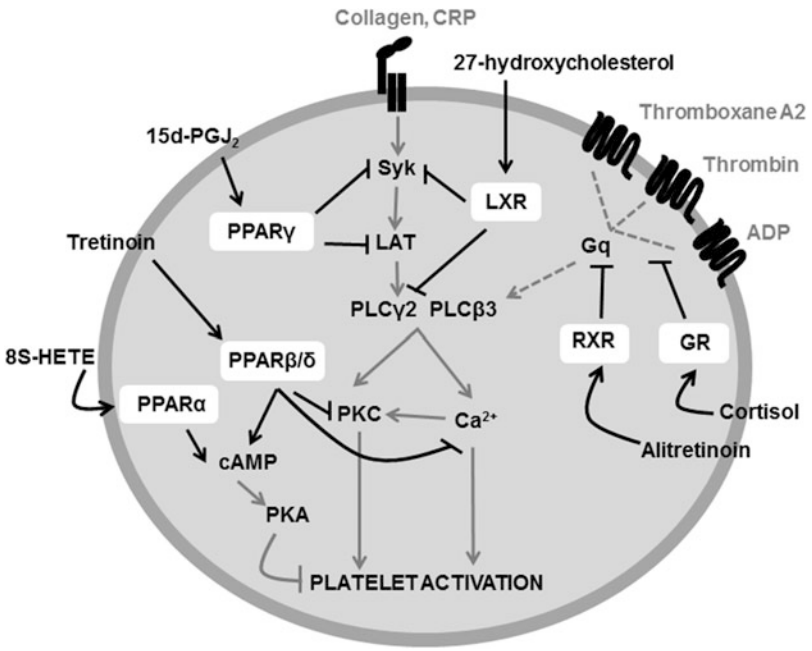

Fig. 2 Intracellular nuclear receptor signaling. Several intracellular nuclear receptors have been identified and characterised in human platelets including the peroxisome proliferator activating receptors (PPAR)s, PPARα, PPARβ/δ and PPARγ, the retinoid X receptor (RXR), liver X receptor (LXR), farnesoid X receptor (FXR) and glucocorticoid receptor (GR). The nuclear receptors are believed to negatively regulate platelet activity through various mechanisms following activation by their ligands, although these mechanisms are not well characterised. Both PPARα and PPARβ/δ are thought to negatively regulate platelet function through regulation of cAMP levels, whilst PPARγ and LXR receptors interact with and inhibit components of early GPVI signaling. In contrast RXR and GR appear to inhibit Gq signaling events. *Grey lines* represent platelet agonist signaling and *black arrows* represent the pathways or proteins that are modulated by nuclear receptor agonists in platelets

Peroxisome Proliferator Activated Receptors

PPARs represent three nuclear receptor isoforms, PPARα, PPARβ and PPARγ (Berry et al. 2003) which are involved in cell development, differentiation, cholesterol and fatty acid metabolism and glucose homeostasis (O'Brien et al. 2007; Barak et al. 1999; Kersten et al. 2000). All three isoforms of PPAR, upon binding to their ligands, are capable of heterodimerising with RXR, another nuclear receptor and all have been identified to have acute non-genomic negative regulatory effects in human platelets.

Treatment of platelets with ligands for PPARα such as fenofibrate and statins was found to increase intracellular levels of cAMP resulting in inhibition of ADP-stimulated platelet activation. Fenofibrate was also found to inhibit platelet activation and increase bleeding time in WT mice but not mice deficient in PPARα$^{-/-}$ (Ali et al. 2009a). Following activation by fenofibrate, PPARα was found to associate with PKCα a key positive mediator of platelet activation, and it is thought that this interaction may in part contribute to the negative regulation of platelet activation that is observed following treatment with PPARα ligands.

PPARβ/δ has been shown to decrease plaque formation and attenuate the progression of atherosclerosis (Lee et al. 2003). Studies using synthetic agonists for PPARβ/δ, GW0742 and L-165041 have identified inhibitory actions for PPARβ/δ ligands on the mobilisation of intracellular Ca^{2+} and platelet aggregation following stimulation by ADP and other platelet agonists (Ali et al. 2006b). PGI_2 a key inhibitory mediator of platelet function is also a ligand for PPARβ/δ and some of its inhibitory effects on platelet activity could be mediated through PPARβ/δ (Forman et al. 1997). As with PPARα, treatment of platelets with agonists of PPARβ/δ results in an increase in cAMP levels and PKCα has been identified as a potential binding partner of the receptor and a potential mechanism by which PPARβ/δ may regulate platelet reactivity (Ali et al. 2009b).

Agonists of PPARγ, the thiazolidinediones are currently in use for the treatment of type 2 diabetes mellitus and have been observed clinically to have cardio-protective properties and reduce the risk of myocardial infarction (Dormandy et al. 2005; Sauer et al. 2006). The emerging role of PPARγ agonists as negative regulators of platelet function may provide a mechanistic basis for this observation. A clinical study that measured platelet function in patients with coronary heart disease treated with rosiglitazone reported long-term anti-platelet effects with down-regulation of P-selectin exposure and granule secretion in treated patients (Sidhu et al. 2004). Treatment of platelets ex vivo with the endogenous agonist of PPARγ, $15dPGJ_2$ or the synthetic agonist rosiglitazone results in reduced platelet responses including granule secretion and thromboxane B_2 (T_XB_2) synthesis in response to thrombin or ADP (Akbiyik et al. 2004). Agonists of PPARγ have also been shown to suppress platelet activation stimulated by GPVI agonists, with platelet aggregation, granule secretion and mobilisation of intracellular Ca^{2+} inhibited following treatment with $15dPGJ_2$ (Moraes et al. 2010c). Treatment with PPARγ agonists results in reduced thrombus formation in vitro and in vivo (Moraes et al. 2010c; Li et al. 2005).

Additionally statins that are routinely prescribed as cholesterol lowering drugs have also been shown to activate PPARs (Ali et al. 2009a). Treatment of human whole blood with the statins, pravastatin, fluvastatin and simvastatin all resulted in a reduction in platelet aggregation to ADP. This decrease in platelet activity was attributed to a PPAR mediated increase in cAMP levels.

RXR

Human platelets and megakaryocytes express RXRα and RXRβ (Moraes et al. 2007). Treatment of platelets with the endogenous agonist of RXR, 9-cis-retinoic acid or the synthetic agonist, methoprene acid, results in an inhibition of Gq protein coupled induced platelet aggregation that is stimulated by ADP and thromboxane A2 (TxA2). It is thought RXR regulates GPCR mediated platelet activation by binding to Gq in a ligand-dependent manner inhibiting Gq induced rac activation and intracellular Ca^{2+} release (Moraes et al. 2007).

LXR

The LXRα and LXRβ isoforms of LXR are implicated in the regulation of fatty acid, cholesterol and glucose homeostasis (Viennois et al. 2011, 2012) and agonists for LXR have been described to have anti-inflammatory effects and be atheroprotective (Joseph et al. 2002; Tangirala et al. 2002). LXRβ has been identified as the isoform present in platelets (Spyridon et al. 2011), and endogenous ligands for the LXR receptors include oxysterols (oxygenated derivatives of cholesterol) and several synthetic agonists including GW3965 and T0901317 also exist (Gabbi et al. 2014; Wójcicka et al. 2015). Treatment of platelets with synthetic agonist GW3965 results in inhibition of platelet activation, including aggregation, secretion and integrin activation stimulated by collagen, CRP or thrombin. GW3965-treated mice form smaller, less stable thrombi following laser injury of the cremaster arterioles. LXR has also been shown to interact with several components of the GPVI signaling pathway following treatment with GW3965, including Syk and PLCγ2, resulting in decreased phosphorylation and signaling (Spyridon et al. 2011; Moraes et al. 2010b).

Glucocorticoid Receptor

The glucocorticoid receptor (GR) is activated by glucocorticoid steroid hormones, a major class of anti-inflammatory hormones, and prednisolone a synthetic derivative of cortisol that has been used to understand the role of GR in the regulation of platelet function. Platelets preincubated with prednisolone prior to agonist stimulation show reduced aggregation and TxB_2 release in response to both ADP and TxA_2 mimetic U46619 which could be reversed following treatment with a GR antagonist mifepristone (Moraes et al. 2005b). However, the mechanism underlying negative regulation of platelet function by GR agonists is still poorly understood.

Negative Regulators of Small GTPases and Integrin $\alpha_{IIb}\beta_3$ Activation

One of the key processes that underpin thrombus formation is the activation of the integrin $\alpha_{IIb}\beta_3$. Activation of $\alpha_{IIb}\beta_3$ results in a conformational change in the receptor that enables fibrinogen binding and aggregation, and also initiates outside in signaling which sustains platelet activation. Suppression of integrin $\alpha_{IIb}\beta_3$ activation prevents inappropriate platelet aggregation and excessive thrombus formation that can cause vessel occlusion (Fig. 3).

RASA3

RASA3 has recently been identified as an important inhibitor of integrin $\alpha_{IIb}\beta_3$ activation (Stefanini et al. 2015) through the regulation of Rap1b which is a critical regulator of integrin function in platelets (Chrzanowska-Wodnicka et al. 2005; Stefanini et al. 2012). Rap1b is positively regulated by the Ca^{2+}-sensing guanine exchange factor (GEF), CAlDAG-GEF1 (Crittenden et al. 2004) and RASA3 provides opposing, negative regulation that maintains platelets in a quiescent state when at resting $[Ca^{2+}]_i$ (Stefanini et al. 2015). RASA3 deficiency is embryonically lethal in mice but homozygous expression of a mutated form of RASA3 with impaired activity results in viable animals with platelet hyperreactivity that can be rescued by simultaneous deficiency of CALDAG-GEF1. However, integrin $\alpha_{IIb}\beta_3$ activation in the double knockout mouse occurs independently of the ADP receptor P2Y12 and PI3K.

JAM-A

JAM-A is a transmembrane protein of the CTX family that is expressed on cell surface and has been identified in platelets. Mice deficient in JAM-A display increased aggregation to several platelet agonists, increased spreading on fibrinogen and clot retraction and increased thrombus formation indicating a role for JAM-A in the negative regulation of integrin $\alpha_{IIb}\beta_3$ (Naik et al. 2012). JAM-A is phosphorylated in resting platelets and associates with $\alpha_{IIb}\beta_3$ (Naik et al. 2012). JAM-A is proposed to keep the integrin inactive by binding C-terminal src kinase (Csk) via its SH2 domain which recruits it to the integrin. Recruitment of Csk ensures that c-Src, which is associated with the integrin, remains in an inactive state through phosphorylation of the inhibitory Y529 in c-Src's regulatory domain (Naik et al. 2014). On platelet activation JAM-A is dephosphorylated, Csk dissociates enabling activation of c-Src and integrin $\alpha_{IIb}\beta_3$ activation.

Studies using ApoE deficient mice that model high plasma cholesterol levels and atherosclerosis have highlighted the importance of the platelet inhibitory receptor JAM-A in the development of the disease state. A recent study has shown that platelet deficiency in the

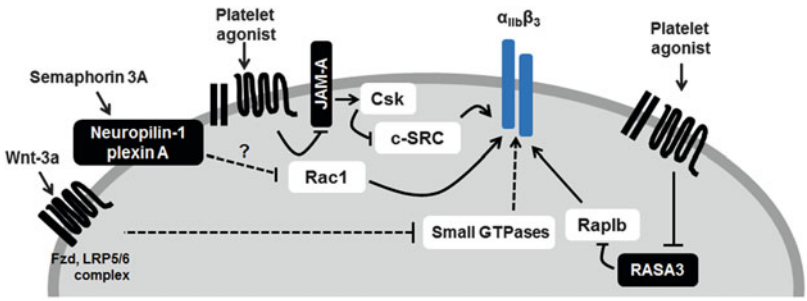

Fig. 3 Negative regulators of small GTPases and integrin $\alpha_{IIb}\beta_3$. RASA3 inhibits Rap1b, preventing signal transduction that enables integrin $\alpha_{IIb}\beta_3$ activation until PI3K activation downstream of Gi-coupled P2Y12 inhibits RASA3. Wnt 3a activates the β-catenin signaling pathway that negatively regulates small GTPases including Rac1 and Rap1 that underpin integrin activation. The neuropilin-1-plexin A complex is activated by semaphorin 3A which also negatively regulates Rac1. JAM-A inhibits c-Src-dependent integrin $\alpha_{IIb}\beta_3$ outside-in signaling through activation of Csk which negatively regulates c-Src. Stimulation with agonists causes dissociation of JAM-A from integrin $\alpha_{IIb}\beta_3$, reducing Csk activity and enabling activation of c-Src mediated integrin $\alpha_{IIb}\beta_3$ outside-in signaling. Semaphorin 3A is believed to activate the neuropilin-1 plexin A complex which inhibits Rac1 causing inhibition of integrin $\alpha_{IIb}\beta_3$ activation

inhibitory receptor JAM-A in ApoE$^{-/-}$ mice fed a high fat diet increases aortic plaque formation and recruitment of inflammatory cells, suggesting that platelet hyperreactivity such as that observed in JAM-A$^{-/-}$ platelets can contribute to atherosclerotic plaque formation (Karshovska et al. 2015).

ESAM

ESAM, a transmembrane glycoprotein, like JAM-A is a member of the CTX family and is also suggested to be involved in the negative regulation of adhesion and integrin $\alpha_{IIb}\beta_3$ outside-in signaling. In contrast to JAM-A, ESAM appears to negatively regulate integrin $\alpha_{IIb}\beta_3$ and limit its activity following platelet activation. ESAM is contained in platelet alpha granules and is translocated to the cell surface on activation (Nasdala et al. 2002). Mouse platelets deficient in ESAM show increased aggregation to GPCR agonists, inhibition of clot retraction, increased thrombus formation in vivo and reduced tail bleeding (Stalker et al. 2009). The mechanism by which ESAM functions is currently unknown, although interaction via its PDZ domain with NHERF-1, a scaffold protein highlights possible interaction with and regulation of several proteins, including GPCRs (Hall et al. 1998), G proteins and PLCβ (Rochdi et al. 2002) and components of the cytoskeleton (Shenolikar et al. 2004).

Wnt3a

Wnt3a is a glycoprotein that is released from endothelial cells (Goodwin et al. 2006) and also from TRAP stimulated platelets, enabling platelets to self-regulate and limit activation (Steele et al. 2009). Treatment of platelets with Wnt3a results in an inhibition of platelet adhesion and shape change, reduced dense granule secretion and reduced RhoA activation leading to diminished integrin $\alpha_{IIb}\beta_3$ activation and aggregation. In platelets, Wnt3a is thought to exert its effects through activation of the canonical Wnt-β-catenin signaling pathway components that also appear to be present in platelets (Semenov et al. 2007; MacDonald et al. 2007; Huang and He 2008). In other cell types β-catenin has been shown to play a role in the regulation of cell adhesion, where it is involved in supporting the interaction of cadherins to the cytoskeleton (Huang and He 2008). Negative regulation of platelet activation via Wnt3a signaling is thought to occur through regulation of small GTPase activity, including Rap1, Cdc42, Rac1, RhoA (Steele et al. 2012). It is thought that by favouring the GDP-bound state of Rap1 and Rho via the regulation of Rap1GAP and RhoA GTPase activity, whilst increasing levels of Cdc42 and Rac1 GTP levels, Wnt3a inhibits integrin $\alpha_{IIb}\beta_3$ adhesion and spreading.

Neuropilin-1-Plexin A Complex

Semaphorin 3A exists as a soluble covalently bound homodimer and is secreted by vascular endothelial cells (Serini et al. 2003). Semaphorin 3A negatively regulates platelet function through binding to the neuropilin-1-plexin A receptor complex which has been identified in platelets (Takahashi et al. 1999; Tamagnone et al. 1999; Kashiwagi et al. 2005). Semaphorin 3A treatment inhibits platelet function, possibly through regulation of integrin $\alpha_{IIb}\beta_3$ as activation of the integrin, aggregation and adhesion and spreading evoked by several platelet agonists are impaired. The exact mechanisms by which Semaphorin 3A inhibits platelet function have not been fully elucidated, although inhibition of the GTPase Rac1 appears to be a key regulatory step in the negative regulation of $\alpha_{IIb}\beta_3$ and cytoskeletal rearrangements (Kashiwagi et al. 2005).

Cyclic Nucleotide Signaling

Cyclic nucleotides cAMP and cGMP are well-established inhibitors of platelet activation. Endothelium derived prostacyclin (PGI_2) and nitric oxide (NO) activate the production of cAMP and cGMP, respectively, and play essential roles in keeping platelets in the resting state in the circulation. The regulation of platelets by these molecules is discussed in detail in Chapter X.

cAMP and Protein Kinase A

PGI_2 binds to and activates the prostaglandin receptor on the platelet surface (Dutta-Roy and Sinha 1987), which then propagates inhibitory signaling through the activation of Gs subunits that activate adenylyl cyclase and stimulate the production of cAMP from ATP (Gorman et al. 1977). cAMP activates cAMP dependent protein kinase A (PKA). Following platelet activation by agonists such as ADP and thrombin, adenylyl cyclase activity is inhibited and cAMP levels reduced through Gi signaling (Jantzen et al. 2001; Yang et al. 2002). PKA phosphorylates multiple target proteins that maintain platelets in an inactive resting state by limiting platelet activation. Several substrates of PKA have been well characterised and are linked to the negative regulation of platelets, including VASP which is involved in cytoskeletal rearrangements (Waldmann et al. 1987; Butt et al. 1994), inhibition of Rap1b a key regulator of integrin $\alpha_{IIb}\beta_3$ affinity (Schultess et al. 2005; Miura et al. 1992), inositol

trisphosphate receptor (IP_3R) which is involved in Ca^{2+} regulation (Cavallini et al. 1996) and the thromboxane A2 receptor (Reid and Kinsella 2003). Other targets also include Gα13 (Manganello et al. 1999), GPIbβ (Wardell et al. 1989) and CALDAG-GEFI (Schultess et al. 2005)

Recent studies have identified a cAMP independent mechanism of PKA activation following stimulation of platelets by thrombin or collagen (Gambaryan et al. 2010). In resting platelets a population of platelet PKA appears to be associated with NFkB-IkBα. Following stimulation by collagen or thrombin the catalytic subunit of PKA dissociates from NFkB-IkBα and is activated, enabling phosphorylation and activation of its substrates. This identifies an inhibitory feedback mechanism that prevents excessive platelet activation in response to stimuli (Gambaryan et al. 2010).

Regulation of cGMP and PKG by Platelet-Derived NO

The cyclic nucleotide cGMP is a key negative regulator of platelet activation that inhibits platelets to keep them in the resting state in the healthy vasculature (Smolenski 2012). Endothelial release of nitric oxide (NO) keeps platelets inactive by regulating their intracellular levels of cGMP (Mellion et al. 1981; Radomski et al. 1987). Following the synthesis and release of NO from the healthy endothelium, NO crosses the platelet plasma membrane and binds to and activates, soluble guanyl cyclase (sGC), leading to the increased production of cGMP from GTP and activation of protein kinase G (PKG). Levels of cGMP are controlled by the phosphodiesterase, PDE5A which is present in platelets (Haslam et al. 1999). Platelets deficient in PKG are insensitive to cGMP-mediated inhibition of intracellular Ca^{2+} release (Eigenthaler et al. 1993) and PKG knockout mice have a prothrombotic phenotype and exhibit increased intravascular adhesion and aggregation following ischaemia (Massberg et al. 1999).

Platelets are now also considered to be a source of NO. The mechanism by which platelets synthesise NO and whether or not it is of physiological importance is, however, still an area of contention. Although NO itself is an established negative regulator of platelet function, the vascular endothelium has traditionally been considered the dominant source of NO within blood vessels. Platelets have been reported to express two nitric oxide synthase (NOS) isoforms, iNOS and eNOS but generate less NO than endothelial cells (Radomski et al. 1990b) and the presence of eNOS is contentious (Gambaryan et al. 2008). Studies into the effects of platelet-derived NO on platelet function using iNOS/eNOS knockout mice have suggested that eNOS is not a major regulator of platelet function (Gambaryan et al. 2008; Tymvios et al. 2009). There is evidence that platelets can produce NO from nitrate although further research is needed to understand the mechanism (Apostoli et al. 2014). Platelet NO production is inducible and is mediated by Ca^{2+} elevation (Radomski et al. 1990b) and is therefore stimulated by many platelet agonists such as ADP and arachidonic acid. However, the question of whether platelet-derived NO can inhibit platelet aggregation is a source of debate (Gkaliagkousi et al. 2007) with some reports describing platelet-derived NO-mediated inhibition (Radomski et al. 1990a, b) and others reporting no effect (Thomas et al. 1990; Thompson et al. 1986). More recent studies have provided further evidence for the presence of functional eNOS in platelets by measuring NO production in single platelets under flow (Cozzi et al. 2015) and also highlighted the anti-thrombotic potential of drugs that modulate platelet eNOS activity (Momi et al. 2014).

Defects in cAMP/cGMP signaling pathways have the potential to contribute to platelet hyperreactivity in cardiovascular disease including ischemic heart disease, heart failure and diabetes where the reduced sensitivity of platelets to the inhibitory effects of NO contributes to platelet hyperreactivity (Chirkov and Horowitz 2007). Platelets from patients with type 2 diabetes mellitus and insulin insensitivity, for example, have reduced sensitivity to NO and prostacyclin and consequently higher platelet reactivity. Additionally a number of individuals with genetic abnormalities in prostacyclin signaling have reduced cAMP levels, resulting in hyper-reactive platelets and a prothrombotic state, and defects in sGC function are linked to an increase prevalence of ischemic heart disease, heart failure and diabetes (van Geet et al. 2009). In contrast patients with hypersensitivity to prostacyclin signaling, as a result of increased activity of Gs proteins show an increased risk of bleeding which is attributed to increased cAMP levels and an increased inhibition of platelet function (van Geet et al. 2009). It is also suggested that in patients with obesity in addition to defects in sGC function, cAMP synthesis may also be altered and defects in downstream effectors of cAMP and cGMP signaling may contribute to platelet hyperreactivity (Russo et al. 2010).

Other Mechanisms of Platelet Inhibition

Phosphatases

Protein modification by phosphorylation is a key mechanism of signal transduction in platelets. Phosphorylation is a reversible post-translational modification that enables regulation of signal transduction and platelet function by phosphatase dependent dephosphorylation of key signaling proteins. Phosphatases are key mediators of several negative signaling pathways in platelets such as those exhibited by

the ITIM containing receptors. Protein tyrosine phosphatases such as SHP1 and SHP2, SHIP1 and SHIP2, PP2, PTEN and TULA2 have well-established roles in the negative regulation of platelet signaling, in general negatively regulating receptor proximal signaling events leading to reduced mobilisation of Ca^{2+}, granule secretion and integrin activation (Senis 2013). SHP2 is well-established for its roles in the negative regulation of platelet signaling events downstream of the majority of platelet agonists, including GPVI and GPCRs (Ma et al. 2012; Jackson et al. 1997). These observations are supported by studies using mouse platelets deficient in SHP2 which show enhanced activation to fibrinogen (Mazharian et al. 2013). The histidine phosphatase TULA2 dephosphorylates and inactivates Syk preventing further downstream signaling (Thomas et al. 2010). TULA2 deficient mice platelets show increased hyperreactivity to GPVI agonists, and also increased thrombus formation and reduced bleeding times. The phosphoinositide phosphatases SHIP1 and PTEN are involved in the regulation of PI3K function through alteration of the phosphoinositol cycle (Laurent et al. 2014). PTEN-deficient platelets which show hyper-responsiveness to collagen exhibit increased PI3K activity and show reduced bleeding times in vivo. Finally the serine/threonine kinase phosphatase PP2A is involved in the negative regulation of integrin $\alpha_{IIb}\beta_3$ function and signaling (Pradhan et al. 2010; Gushiken et al. 2008). PP2A mediates dephosphorylation of PKCζ and PTP-1B which reduces Src phosphorylation and activation (Mayanglambam et al. 2011; Pradhan et al. 2010; Gushiken et al. 2008). It is important to note, however, that whilst several phosphatases are associated with negative regulation of platelet function, many positive regulatory functions for phosphatases have been identified in platelets (Senis 2013).

Receptor Desensitisation

Platelets can regulate and limit their responsiveness to platelet agonists, through desensitisation of their receptors following agonist stimulation. ADP, for example, is a critical secondary mediator of platelet signaling, yet despite this it has been shown that platelets desensitise following continued exposure to ADP (Mundell et al. 2006; Hardy et al. 2005; Cunningham et al. 2013). This diminished response is attributed to desensitisation of both the P2Y1 and P2Y12 receptors via agonist mediated internalisation of each receptor. Studies using both platelets and 1321N1 cells have identified that the P2Y1 and P2Y12 receptors are desensitised by different mechanisms following platelet agonist stimulation. Desensitisation of the P2Y1 receptor is mediated by both classical and novel isoforms of protein kinase C (PKC), whilst P2Y12 desensitisation is mediated by G protein coupled receptor kinase and the novel isoforms of PKC (Hardy et al. 2005).

PKC Isoforms

Protein kinase C (PKC), a family of serine/threonine kinases regulates many aspects of platelet signaling. The different isoforms of PKC are classified into three different subtypes classical, novel and atypical isoforms, according to their structure and mechanism of regulation. Several isoforms have been identified in human platelets although expression of some isoforms is controversial (Newton 1997; Mellor and Parker 1998; Murugappan et al. 2004; Buensuceso et al. 2005; Hall et al. 2008; Pears et al. 2008; Konopatskaya et al. 2009; Bynagari et al. 2009; Nagy et al. 2009; Harper and Poole 2010). Historically the PKC family were considered to play an overall positive role in the regulation of platelet activity, as broad spectrum PKC inhibitors were shown to inhibit granule secretion, TxA_2 synthesis, integrin activation, aggregation and thrombus formation (Harper and Poole 2010). However, negative regulatory roles have subsequently also been identified for the PKC family. Studies using broad spectrum inhibitors have also identified negative roles in the regulation of receptor desensitisation (see section on receptor desensitisation) and Ca^{2+} release, and studies using isoform specific deficient mice have identified negative regulatory roles for the novel isoforms PKCδ and PKCθ (Harper and Poole 2010).

Transgenic mice deficient in PKCδ have identified a negative regulatory role for PKCδ in the regulation of integrin $\alpha_{IIb}\beta_3$ outside-in signaling and filopodia formation on fibrinogen due to an interaction between PKCδ and VASP (Pula et al. 2006). PKCδ has also been shown to negatively regulate GPVI-induced platelet responses, as platelets from PKCδ deficient mice have enhanced aggregation and dense granule secretion in comparison to WT controls. This was also confirmed in human platelets using a cell permeable peptide that is designed to block the interaction of PKCδ and δ(V1-1)-TAT (Chari et al. 2009). Contrasting reports, however, also exist as other groups found no abnormality in GPVI-dependent dense granule secretion in $PKC\delta^{-/-}$ platelets (Pula et al. 2006). PKCδ deficient platelets generate larger thrombi when measured in vitro but not when measured in vivo (Gilio et al. 2010; Chari et al. 2009). The overall role of PKCδ has therefore been difficult to define, possibly as a consequence of diverse positive and negative regulatory roles played by this PKC isoform.

Studies that have utilised PKCθ isoform-specific inhibitors have identified negative roles for PKCθ in several processes in GPVI-induced platelet activation, including aggregation, α-granule secretion, $\alpha_{IIb}\beta_3$ activation and changes in intracellular Ca^{2+} levels, as all were increased following treatment of platelets with the inhibitor. Studies that have utilised PKCθ deficient mice have generated conflicting reports of both positive and negative roles in the regulation of platelet activation (Hall et al. 2008; Nagy et al. 2009; Harper and Poole 2009, 2010; Cohen et al. 2011;

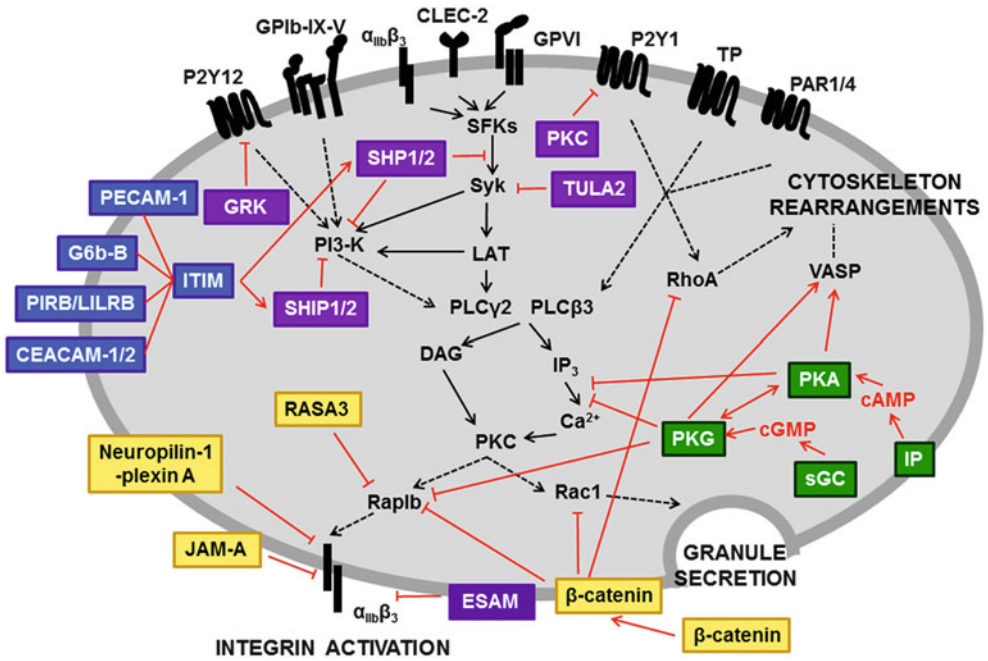

Fig. 4 Platelet-derived inhibitors of platelet function. Well-characterised activatory pathways (*black*) are regulated by multiple inhibitory pathways that regulate platelet responses to support their role in hemostasis. Receptors that signal through ITIMs (*blue*) recruit SHP1/2 and SHIP1/2 in close proximity to ITAM receptors where they dephosphorylate components of activation pathways, inactivating them to provide negative regulation. Small GTPases regulate several important process in platelets such as granule secretion and integrin $\alpha_{IIb}\beta_3$ activation and so inhibitors of small GTPases (*yellow*) such as RASA3 and Wnt3a/β-catenin provide negative regulation for these key events. PKA and PKG are inhibitory kinases that are regulated by cyclic nucleotides (*green*), although these pathways are primarily regulated by non-platelet-derived inhibitors they are also potentially regulated by platelet-derived NO and non-cAMP dependent PKA activation. Other negative regulators (*purple*) such as phosphatases and kinases that regulate receptor desensitisation do not easily fit into discrete categories and mediate negative feedback via diverse mechanisms

Unsworth et al. 2012; Gilio et al. 2010). These differences have been attributed to different experimental conditions whereby PKCθ may have a negative role following exposure to low agonist concentrations, and a positive role following exposure to higher concentrations of platelet agonists (Hall et al. 2008; Nagy et al. 2009; Cohen et al. 2011).

Conclusion

Platelet-derived mediators of negative regulation all function to limit or restrict platelet activation, yet are mechanistically diverse and affect pathways involved in many different processes (Fig. 4). While many negative regulators have been identified, many of the processes that they regulate to achieve platelet inhibition are not yet fully characterised. However, it is becoming increasingly clear that negative regulatory mechanisms rival the complexity of the positive regulators of platelet activation. The key challenge in the field of inhibitory platelet signaling will be to establish the physiological and pathological importance of these proteins, their potential as drug targets and their role in determining disease risk.

Take Home Messages

- Platelet activation is triggered by stimuli that arise within the circulation
- Activated platelets initiate their own positive feedback mechanisms that support thrombus growth
- Platelets also generate negative feedback signals that can limit thrombus development
- The mechanisms of negative regulators are diverse and many have not yet been fully characterised
- Negative regulatory mechanisms may be as important as positive regulators to understand disease risk and discover new drug targets and therapies

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Part II

Methodology

Platelet Function Tests

Marie Lordkipanidzé and Paul Harrison

Abstract

Many platelet function assays have been developed over the last century in an attempt to capture the natural ability of platelets to form aggregates in response to vascular injury. From physiological assays, such as the bleeding time, to diagnostic assays used within specialized hematology departments, to point-of-care assays that are intended as clinical decision aids on wards and in operating rooms, and finally to high-throughput deep phenotyping assays intended for precision medicine, platelet function assays have become increasingly commonplace in many settings. This chapter presents an overview of the most commonly used platelet functions assays and discusses important variables to take into account when performing platelet function testing, ranging from pre-analytical issues to the clinical utility of individual tests.

Introduction

Many platelet function assays have been developed over the last century in an attempt to mimic the natural ability of platelets to form aggregates in response to vascular injury. From physiological assays, such as the bleeding time, to diagnostic assays, such as those used to this day in specialized hematology departments, to point-of-care assays that are intended as clinical decision aids on wards and in operating rooms, and finally to high-throughput deep phenotyping assays intended for precision medicine, platelet function assays have become increasingly commonplace in many settings. The continued interest in platelets and their contribution to health and disease continuously drives innovation in this field, tackling the challenges of working with live, dynamic, and sensitive cells.

In order to accomplish their many roles, not only in maintaining hemostasis, but also in inflammation, host defense and immunity, wound healing, fetal vascular remodeling, tumor growth and metastasis, liver disease, and angiogenesis, to name a few, platelets are a treasure trove of membrane receptors and anchoring proteins, diverse granular contents, and *de novo*-generated mediators (Nurden 2011; McFadyen and Kaplan 2015). These need to intervene in a coordinated fashion to ensure appropriate platelet activation, degranulation and aggregation, as any imbalance may lead to either bleeding or thrombotic events. Because of this biological complexity, with many redundant activation pathways working in concert, studying platelet function can be difficult and requires specialized methods to capture this diversity of platelet responses.

In this chapter, we discuss general principles in platelet function testing, with special attention to pre-analytical variables, and highlight areas of standardization where guidelines are available. We also present an overview of currently available platelet function assays and their clinical usefulness. Specific techniques of platelet flow cytometry (Carubbi et al. 2017) and platelet aggregometry (Hayward

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and Moffat 2017) are detailed further in the following sections, along with applications including monitoring of antiplatelet therapy (Tantry et al. 2017) and diagnosis of platelet defects (Mezzano and Pereira 2017).

Bleeding Time: A Physiological Approach to Platelet Function Testing

The first assessment of platelet function to be successfully used was initially described as a coagulation test by M.G. Milian in 1901 (Milian 1901). Shown to be correlated with platelet dysfunction by W.W. Duke in 1910 (Duke 1910), the test underwent further refinement at the hands of A. C. Ivy in 1941 and has become known as the Duke-Ivy Bleeding Time (Ivy et al. 1941). In its most accepted form, the assay is carried out on the ventral surface of the forearm where skin thickness is uniform, with a blood pressure cuff applied to the upper arm and inflated to 40 mmHg. The technique consists of recording the time required for a blood clot to form at the site of a 5 mm long by 1 mm deep longitudinal incision, and the test ends when the flow of blood is stopped. Commercial spring-loaded devices containing sterile blades (e.g., Simplate II® by the Organon Teknika Corporation and later Surgicutt® by the International Technidyne Corporation) have further standardized the procedure, which has remained the most useful screening test of platelet function until the early 1990s (The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force 1988). This assay has brought forward a new understanding of platelet function in vivo, including the effect of platelets on hemostasis, the interaction of platelets with vascular endothelial cells and other blood elements, co-activation of platelets and the coagulation system, platelet release reactions in vivo, and various platelet diatheses (Gresele et al. 1987, 2003; Kyrle et al. 1987; Michelson et al. 1991; Weiss and Lages 1993; Weltermann et al. 1999; Ciferri et al. 2000; Undas et al. 2001; Falcinelli et al. 2007; Lubsczyk et al. 2010; Giannini et al. 2011; Traby et al. 2016).

Among the advantages of this assay are its simplicity and its accessibility, as no expensive equipment or specialized laboratory is required. This ensured the test's widespread use in and outside of specialized centers for decades. The Duke-Ivy Bleeding Time measures physiological hemostasis driven by platelets and plasma adhesive proteins such as von Willebrand factor (VWF), as well as the interplay of blood components with the vessel wall. For this reason, it cannot pinpoint the origin of the defect, but an abnormally prolonged Bleeding Time is indicative of a hemostatic deficiency.

Several factors have led to the downfall of the Bleeding Time as a screening assay, in favor of novel platelet function tests. Chiefly, they included the poor reproducibility despite the use of standardized devices, the invasive nature of the test which could lead to scarring, and the relative insensitivity of the Bleeding Time to many mild platelet defects. In the early 1990s, several investigators concluded that an accurate bleeding history was a more valuable screening test, as it captured mild bleeding tendency more robustly than the Bleeding Time (Rodgers and Levin 1990; Lind 1991; Peterson et al. 1998). As a result, widespread use of the bleeding time has rapidly declined over the last 20 years, to be replaced by other less invasive platelet function assays carried out ex vivo on freshly collected blood samples (Harrison et al. 2011).

Important Considerations in Platelet Function Testing

Most platelet function assays have strict requirements in terms of blood drawing and sample preparations. Recent surveys have highlighted that platelet function testing practices can vary significantly between laboratories (Moffat et al. 2005; Jennings et al. 2008; Cattaneo et al. 2009; Gresele et al. 2014). These differences may explain some of the discrepancies reported between laboratories and emphasize the importance of standardized laboratory approaches. Because platelets can be artifactually activated by inappropriate sample handling, important pre-analytical variables must be taken into consideration before platelet function testing can be performed. Failure to apply some of these key considerations may lead to spurious results (Harrison et al. 2011). This is particularly important, as there are no widely available internal or external quality control materials for platelet function testing (Favaloro 2009), with the exception of the PFA-100/200® device where quality controls have been proposed (Favaloro 2013; Favaloro and Bonar 2014). For this reason, most assays are performed side-by-side with a fresh blood sample from a drug-naïve healthy volunteer, which ensures the viability of reagents and appropriate assay settings (Harrison and Lordkipanidzé 2013). Alternatively, some laboratories derive local reference ranges from a cohort of healthy volunteers, and carry out their platelet function testing according to rigorous standard operating procedures, to limit operator-induced variability. Finally, cartridge-based point-of-care assays have built-in electronic control measures that limit inappropriate test procedures, but pre-analytical conditions may still lead to aberrant test results. The main key pre-analytical considerations are detailed below.

Physiological and Medical Conditions

A number of physiological conditions may influence platelet function results. These include circadian rhythms (Hartley 2012), strenuous exercise (Davis et al. 1990), fasting (Ahuja

et al. 2009), coffee and caffeine-containing beverage consumption (Varani et al. 2000; Natella et al. 2008), and smoking (Rival et al. 1987). Although guidelines in the past have suggested that platelet function studies should only be performed on samples obtained from individuals who were fasting and resting, and who refrained from smoking, caffeine ingestion, and rigorous exercise on the day of testing, pragmatically many of these conditions are hard to control and only have a minor influence on platelet function results (Kottke-Marchant and Corcoran 2002; Harrison et al. 2011; Cattaneo et al. 2013). However, it is necessary to ensure that subjects have refrained from medication or substances known to affect platelet function (which include non-steroidal anti-inflammatory drugs, antiplatelet agents, phosphodiesterase inhibitors, certain psychotropics, and herbal remedies) for 10–14 days, to account for normal platelet turnover (Kottke-Marchant and Corcoran 2002; Harrison et al. 2011; Lordkipanidzé 2012; Cattaneo et al. 2013). This washout period can be longer in case of drugs with a long effective half-life, such as the antiplatelet drug vorapaxar which may take weeks or even months to wear off (Gurbel et al. 2011). An unexpected finding in platelet function results should prompt investigations into these potential confounders, and repeat testing on a fresh blood sample under more suitable conditions may be warranted.

In some instances, deferring platelet function testing or stopping antiplatelet medications cannot be considered. For example, in the context of acute coronary syndromes or in patients requiring chronic antiplatelet therapy as secondary prevention of acute thrombotic events, platelet function testing must be carried out promptly and on-treatment. A number of expert opinion documents have been published with recommendations in terms of interpretation of platelet function results in this context (Michelson et al. 2005; Pulcinelli and Riondino 2006; Kuliczkowski et al. 2009; Tantry et al. 2013; Aradi et al. 2014), and these are treated in more detail in a dedicated section in this book (Tantry et al. 2017).

Venipuncture

Blood should be collected by an experienced phlebotomist, from the antecubital vena fossa, applying a standardized, atraumatic protocol of a clean venipuncture using minimum tourniquet pressure (Harrison et al. 2011). As shear is an important inducer of platelet activation, needles or butterfly cannulae of 19–21 gauge should be preferred, in conjunction with either an evacuated tube system or plastic syringe. A discard tube of 5 ml should be drawn first to avoid tissue factor-induced aggregation from the venipuncture, but this practice is rarely applied in clinical centers with little to no impact on the quality of the blood sample (Favaloro et al. 2008). Underfilling or overfilling of collection tubes is a frequently encountered problem. Collection tubes should be filled to 90 % capacity or to the manufacturer-specified mark, to avoid incorrect sample dilution/anticoagulation which can negatively impact platelet function tests (Favaloro et al. 2008). Samples should be handled gently and inverted three to six times to provide adequate mixing of test sample with the anticoagulant.

Anticoagulants

The most commonly employed anticoagulant for platelet function testing is sodium citrate (Harrison et al. 2011). Other commonly used anticoagulants include acid-citrate-dextrose (ACD), the potent thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK), hirudin, heparin, and the dual thrombin/factor Xa inhibitor benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamide (BAPA). There is no consensus on the best anticoagulant for platelet function testing, and most assays can be performed in samples anticoagulated with any of these agents (Kaiser et al. 2012). Particular precautions must be taken to avoid EDTA-containing tubes, and whenever these are required (e.g., for full blood counts), these should be collected last to avoid potential contamination by carryover (Favaloro et al. 2008).

Specimen Processing

Blood samples collected for platelet function testing should ideally be left to rest for 30 min at room temperature (20–25 °C). Placing samples in a refrigerator/ice pack or in a warm water bath prior to platelet function testing negatively impacts platelet responses (Harrison et al. 2011). Ideally, samples should be collected close to the laboratory, but if transport is necessary, it is important to avoid vibration, shaking, vortexing, or agitation. For this reason, pneumatic tube systems are considered inappropriate for transporting platelet function testing specimens (Thalen et al. 2013). The time delay between collection, transport and analysis should ideally be between 30 min and 2 h, but should not exceed 4 h (Harrison et al. 2011).

For platelet function assays that require the preparation of platelet-rich plasma (PRP), whole blood samples should be centrifuged without using a brake at 200 g for 10 min and at room temperature (Cattaneo et al. 2013). These conditions were shown to result in high quality PRP, by reducing contamination with other blood cells and optimizing platelet reactivity (Femia et al. 2012). Once the PRP has been removed by gentle pipetting, the remaining portion of the blood sample can be centrifuged at room temperature at 1500 g for 15 min to prepare platelet-poor plasma (PPP) (Cattaneo et al. 2013). There is no need to adjust platelet

count in PRP with autologous PPP to a standardized value, as this practice induces extra variability to platelet function results (Cattaneo et al. 2007; Linnemann et al. 2008).

Platelet Function Assays

An impressive array of platelet function assays are available to platelet researchers (Table 1). Many require dedicated skill, expertise, and machinery to run and to interpret, but increasingly, point-of-care instruments are being developed with the intent of applying this technology outside of specialized laboratories. The more commonly used assays are described in more detail below.

Light Transmission Aggregometry

First described by Gustav Born in 1962 and considered by many as the historical gold standard in platelet function testing (Born 1962), light transmission aggregometry (also known as optical aggregometry) measures the increase in light transmission through a PRP sample as platelets aggregate in response to an external stimulus. Addition of a platelet agonist induces the platelets to activate and clump together, which decreases the turbidity of the sample and results in greater light transmission (autologous PPP being used to define 100 % light transmission). The aggregometer captures this dynamic interaction of platelets with each other in real time, resulting in a time-dependent platelet aggregation curve (described in further detail in Section 2.4). An adaptation of this technology allows for luminescence to be read concomitantly to light transmission. By using a luciferin-luciferase substrate added to PRP, a measurement of dense granule secretion can then be performed in parallel with platelet aggregation (Pai et al. 2011) (described in further detail in Wersäll et al. 2017).

This assay offers several advantages. It is very flexible, as there is no restriction to the platelet agonists or agonist concentrations that can be used to stimulate platelets (Hayward et al. 2010; Harrison et al. 2011). The real-time nature of the assay also provides with kinetic information of platelet–platelet interactions; this includes initial platelet shape change, degranulation, and reversible or irreversible aggregate formation (Cazenave et al. 2004). These factors have contributed to making the assay the preferred platelet function test in most specialized laboratories over the world for the better part of the last century (Cattaneo et al. 2013).

Among the disadvantages of the assay, it is important to note that LTA is carried out in PRP, making it relatively non-physiological, stirred at low shear conditions not representative of arterial shear rates where platelet activation normally occurs, and platelets in this assay only form aggregates after a platelet agonist has been added. These conditions do not entirely mimic platelet adhesion, activation and aggregation as they would occur in response to damage to the vessel wall (Harrison and Lordkipanidzé 2013). It is also a time-, labor-, and blood-volume intensive technique. To run a full panel of agonists, conventional LTA requires large blood volumes and a dedicated and experienced operator (Dawood et al. 2012). Interpretation of tracings can also be challenging, which often limits its use to specialist tertiary centers that can maintain their expertise through a high patient volume. This has triggered several attempts at automation of LTA (Lawrie et al. 2014), as well as development of new, and faster approaches to LTA (Sun et al. 2001; Moran et al. 2006; Chan et al. 2011) that are described below.

96-Well Plate-Based Light Transmission Aggregometry

The requirement for a dedicated aggregometer has hampered the availability of LTA in many laboratories. However, as modern 96-well plate readers capable of recording light transmission through multiple samples within a few minutes have become widely available in standard laboratories, several groups have attempted to adapt LTA principles to a high-throughput 96-well plate design (Bednar et al. 1995; Sun et al. 2001; Moran et al. 2006; Chan et al. 2011). This requires the coating of wells with platelet agonists, either in solution or lyophilized, to trigger platelet aggregation after PRP is added to each well with a multichannel pipette. The plate is quickly transferred to a heater-shaker that can maintain the samples at 37 °C and stir the samples to facilitate platelet–platelet interactions. Depending on the plate reader setting, light transmission can be read either at set intervals (e.g., 15 s) or at the end of a fixed period (e.g., 5 min), thus providing either kinetic curves similar to LTA, or an endpoint assessment of platelet aggregation (Chan et al. 2011; Chan and Warner 2012). Similarly to LTA, light transmission is converted into % aggregation by measuring light transmission in PRP and autologous PPP samples.

Despite the fact that various versions of this technique are in use in research laboratories, the experience in clinical settings remains limited. In a head-to-head comparison of the assay with LTA in a group of patients with idiopathic bleeding, the assay was shown to be a promising tool for the screening of bleeding disorders and monitoring of antiplatelet drugs (Lordkipanidzé et al. 2014). Its main use presently is as a tool for a better understanding of basic pathophysiology of platelets (Mylotte et al. 2012; Cooke et al. 2013; Lordkipanidzé et al. 2014).

Table 1 Non-exhaustive list of commonly used platelet function assays

Name of test	Principle	Advantages	Disadvantages	Frequency of use
Global assays of hemostasis				
Bleeding time	In vivo cessation of blood flow	In vivo test, physiological POC	Insensitive, invasive, scarring, high CV	Was widely used, now less popular
Thromboelastography (TEG® or ROTEM®)	Monitoring of rate and quality of clot formation	Global whole blood test	Measures clot properties only, largely platelet-independent unless platelet activators are used	Widely used in surgery and trauma
TEG-6S	Monitoring of rate and quality of clot formation	POC	POC equivalent to TEG or ROTEM	No clinical experience
Electron microscopy	Ultrastructural analysis of platelets	Diagnostic Whole mount technique useful for dense granular imaging	Expensive, specialized equipment	Only available in special units
Platelet activation-based assays				
Flow cytometry	Measurement of platelet glycoproteins and activation markers by fluorescence	Whole blood test, small blood volumes, wide variety of tests	Specialized operator, expensive, samples prone to artifact unless carefully prepared	Frequently used either in core laboratories or as benchtop instruments in research labs
VASP	Flow cytometric or ELISA measurement of phosphorylation of VASP	Measurement of $P2Y_{12}$ receptor signaling	Insensitive to intermediate inhibition of $P2Y_{12}$	Increasing use
Platelet aggregation-based assays				
Light transmission aggregometry (LTA)	Low shear platelet-to-platelet aggregation in response to classic agonists	Gold standard	Time-consuming, sample preparation, poorly standardized	Widely used in specialized labs
96-well-plate based assays in PRP	Based on LTA principles	Lower blood/PRP volumes than LTA. Many replicates and dose–response curves possible	Little widespread experience	Little widespread experience
Plateletworks®	Platelet counting pre- and post-activation	Rapid, simple, POC, small blood volume	Indirect test measuring count after aggregation	Used in surgery and cardiology
VerifyNow®	Fully automated platelet aggregometer to measure antiplatelet therapy	Simple, POC, 3 test cartridges (aspirin, $P2Y_{12}$, and $\alpha_{IIb}\beta_3$)	Inflexible, cartridges can only be used for single purpose	Widely used
WBA	Monitors changes in impedance in response to classic agonists	Whole blood test, multichannel version available	Dependent on platelet count, older instruments require electrodes to be cleaned and recycled	Widely used in specialized labs although less than LTA
Shear-based assays				
PFA-100/200®	High-shear platelet adhesion and aggregation during formation of a platelet plug	Whole blood test, high shear, small blood volumes, simple, rapid, POC, 3 test cartridges (CEPI, CADP and INNOVANCE P2Y)	Inflexible, VWF-dependent, Hct-and platelet count dependent	Widely used
Microfluidic devices	Miniaturized multichannel devices	Whole blood, real-time thrombus formation	Little widespread experience	Research only at present
Assays measuring platelet release reactions				
Lumi-aggregometry	Combined WBA or LTA and nucleotide release	Monitors release reaction with secondary aggregation	Semiquantitative	Widely used in specialized labs, although less than LTA
Adenine nucleotides	Measurement of total and released nucleotides by luminescence or HPLC	Sensitive	Sample preparation, assay calibration, extra equipment	Restricted to specialized labs

(continued)

Table 1 (continued)

Name of test	Principle	Advantages	Disadvantages	Frequency of use
Soluble platelet release markers and sheddome (e.g., serotonin, PF4, βTG, sCD40L, sCD62P, GPV, and GPVI)	Usually by ELISA, also available through bead-based flow cytometry	Relatively simple	Prone to artifact during blood collection and processing	Fairly widely used in research
Serum thromboxane B_2	Immunoassay	Dependent upon platelet COX-1 activity	Prone to artifact	Widespread use
AspirinWorks®	Immunoassay of urinary 11-dehydrothromboxane B_2	Measures stable thromboxane metabolite, dependent upon COX-1 activity	Indirect assay, not platelet-specific, renal function-dependent	Increasing use

COX-1 cyclooxygenase 1, *CV* coefficient of variation, *ELISA* enzyme-linked immunoassay, *GP* glycoprotein, *Hct* hematocrit, *HPLC* high-performance liquid chromatography, *LTA* light transmission aggregometry, *PFA-100/200®* platelet function analyzer 100/200, *PF4* platelet factor 4, *POC* point of care, *PPP* platelet-poor plasma, *PRP* platelet-rich plasma, *sCD40L* soluble CD40 ligand, *sCD62P* soluble CD62P (P-selectin), *βTG* β-thromboglobulin, *VASP* vasodilator-stimulated phosphoprotein, *WBA* whole blood aggregometry

Whole Blood Aggregometry

As its name suggests, whole blood aggregometry (WBA) measures platelet aggregation as it occurs ex vivo, in whole blood. The technique is based on electrical impedance resulting from aggregate formation onto two electrodes immersed in saline-diluted whole blood stimulated with platelet agonists (Cardinal and Flower 1980). There are currently two manufacturers of whole blood aggregometers. The Chronolog apparatus is a two- to four-channel computerized aggregometer, which is compatible with both a disposable single-use set of electrodes and reusable electrodes that require thorough and careful cleaning between uses. The more commonly used instrument in clinical laboratories is the Multiplate® analyzer, mainly because it is semi-automated, highly standardized and uses disposable cuvettes/electrodes with preselected agonists for different applications, including diagnosis of bleeding and monitoring of antiplatelet therapy (Solomon et al. 2011; Valarche et al. 2011; Aradi et al. 2014).

The assay's advantages include performance in whole blood, thus eliminating potential artefactual activation of platelets during the PRP preparation steps; relative ease of use and rapidity, as compared with LTA; the compatibility with a large array of platelet agonists at various concentrations, thus allowing for a better understanding of various activation pathways; and the smaller blood volume requirement as opposed to LTA (Aradi et al. 2014; Kong et al. 2015). However, the assay is influenced by a number of factors, which include platelet count and hematocrit, the anticoagulant used, and the delay between blood sample collection and platelet function testing (Stissing et al. 2011; Kaiser et al. 2012; Rubak et al. 2012; Wurtz et al. 2014). Its use in the clinic is mostly centered on monitoring of antiplatelet therapy (Tantry et al. 2013; Aradi et al. 2014), as there is little published data on the assay's usefulness in investigating inherited platelet disorders (Albanyan et al. 2015).

VerifyNow®

The VerifyNow® system is perhaps the most commonly used point-of-care assay. It is specifically developed to monitor antiplatelet therapy, with dedicated cartridges to assess the effect of aspirin, $P2Y_{12}$ ADP receptor inhibitors, and GPIIbIIIa antagonists. Its ease-of-use, speed, and requirement for little to no technical expertise have made this assay highly popular in settings where platelet function testing could be used to guide antiplatelet therapy, such as in cardiac catheterization laboratories. However, its clinical predictiveness has been questioned lately in view of large clinical trials using the VerifyNow® $P2Y_{12}$ assay to guide antiplatelet therapy having failed to provide clinical benefit (Siller-Matula et al. 2015). Notwithstanding, consensus documents still recommend the use of the VerifyNow® $P2Y_{12}$ assay, as a potential predictor of future cardiovascular and bleeding events (Aradi et al. 2014).

Platelet Function Analyzer (PFA-100/200®)

The recently updated PFA-200® instrument is a cartridge-based assay. It was intended to provide an in vitro equivalent to the bleeding time, and its mode of action requires blood to be aspirated through an aperture in a membrane coated with platelet agonists, and to record the time for a platelet plug to occlude the aperture which is reported by the system as "closure time" (Favaloro and Bonar 2014). Three cartridges are currently available; the CADP cartridge contains collagen and ADP, the CEPI cartridge contains collagen and epinephrine and the INNOVANCE P2Y cartridge contains ADP and PGE_1 supplemented with calcium, with a smaller aperture (100 μm vs 150 μM) (Favaloro 2008; Koessler et al. 2011). The INNOVANCE P2Y cartridge is significantly more sensitive to P2Y12 receptor inhibition than the CADP cartridge and has shown promise in monitoring of antiplatelet drugs as well as in evaluation of congenital

$P2Y_{12}$ receptor defects (Koessler et al. 2011, 2012; Edwards et al. 2012; Scavone et al. 2014).

The assay has gained popularity as a general screening tool for hemostatic disorders due to its ease of use, speed, small blood volume requirement, and the need for little to no specialist training. It is also the only platelet function assay where an external quality control program is available (Favaloro 2009; Favaloro and Bonar 2014). However, a number of factors are known to affect the assay's performance, and these must be taken into account when interpreting the results. Hematocrit and platelet count influence closure time, which makes the assay not suitable for patients with a platelet count below 50×10^9/L or hematocrit below 25 % (Carcao et al. 2002). Patients with non-O blood groups have shorter closure times than blood group O patients, potentially requiring adapted reference ranges that take into account the patient's blood group (Cho et al. 2008). The concentration of sodium citrate used to anticoagulate the sample also influences the results, with 3.8 % giving greater stability of results (Jilma 2001). The high dependence of the assay on von Willebrand factor (due to the high-shear conditions when blood is aspirated through the aperture) makes it an interesting assay for screening of von Willebrand disease, but makes it unsuitable for platelet function testing in this cohort (Favaloro 2002, 2008).

Recent guidelines have suggested that the assay could be used as a screening test to rule out a significant platelet defect in a patient whose clinical history for bleeding is unlikely to point to an inherited platelet disorder, but should not be used as evidence of absence of a platelet defect in patients at high suspicion of inherited platelet dysfunction (Hayward et al. 2006; Harrison et al. 2011).

Thromboelastography® (TEG®)

Thromboelastography® (TEG) and Rotational TEG (ROTEG® or ROTEM®) are similar technologies that assess the hemostatic function as a whole, from thrombus formation to lysis (Luddington 2005; Chen and Teruya 2009). The instruments differ slightly, but the methodology is the same, as a whole blood sample is stirred in a cup with a suspended pin. In anticoagulated samples, the motion between the cup and the pin is unaffected. However, as clot formation is triggered by addition of coagulation or platelet activators, the motion of the cup/pin is hampered, and this translates into a curve depicting clot strength. Both re-calcified plasma and whole blood can be used in this assay, with activators of the tissue factor or contact factor pathways (Chen and Teruya 2009). Arachidonic acid and ADP can also be used as agonists to pre-activate platelets within the TEG system (PlateletMapping™ technology).

The TEG®/ROTEM® tests are well established, mostly in the context of assessing global hemostasis for the management of bleeding and thrombotic risk in surgical, cardiovascular, and trauma patients despite a relatively weak clinical evidence-base (Kozek-Langenecker et al. 2013; Hunt et al. 2015). They are, however, labor-intensive, which has prompted the development of a novel point-of-care microfluidic assay named TEG-6S (Gurbel et al. 2016). It provides all the same test results as traditional TEG, but is a cartridge-based point-of-care rapid analyzer. How this new assay compares with traditional TEG, and whether it will provide clinically meaningful results remains to be established.

Flow Cytometry

Important technological advances have made flow cytometers available not only in specialized core laboratories, but also as benchtop instruments in non-dedicated laboratories. A vast array of antibodies coupled with fluorochromes is commercially available, specifically targeted against individual platelet proteins, granules, and lipid membranes. Flow cytometry is therefore a powerful and popular tool to study many aspects of platelet biology and function, and is described in more detail in Carubbi et al. (2017).

Assessment of platelet function by flow cytometry can be performed on isolated platelets, but the use of diluted anticoagulated blood is preferred. The most common markers of platelet activation used in the literature are P-selectin expression on the platelet surface as a marker of α-granule secretion; the conformational change in integrin $\alpha_{IIb}\beta_3$ into its active state with the PAC-1 antibody; and phosphorylation of vasodilator-stimulated phosphoprotein [VASP] as a marker of $P2Y_{12}$ receptor activation-dependent signaling (Schmitz et al. 1998; Matzdorff 2005). However, the field is in constant progress, especially with novel multiplex technologies that allow for the concomitant measurement of multiple analytes in blood samples as small as a few microliters (Spurgeon et al. 2014). Moreover, the assay is being actively developed for assessing platelet function in patients with thrombocytopenia, as it is performed independently of platelet count (Frelinger et al. 2015). Efforts have been made to standardize the use of flow cytometry for platelet function testing (Schmitz et al. 1998; Lee et al. 2008), but it remains a challenging assay to homogenize, which makes comparisons between laboratories difficult. More recently, reagents have become available so that remote activation and fixing can be performed reliably before analysis within a central core laboratory (Dovlatova et al. 2015).

Conclusion

The last century has seen many developments in platelet function testing and has brought forward a better understanding of platelet biology, physiology, and pathology (Coller 2011). The time when functional assessment of platelets was confined to specialized laboratories and required dedicated equipment and personnel has given way to point-of-care, near-patient instruments of platelet function assessment and changed the way platelet studies are conducted (Harrison and Lordkipanidzé 2013). Today's challenges in platelet function testing include determining the true clinical usefulness of these assays for predicting thrombosis and bleeding, and the development of individualized approaches to mitigate risks in individual patients (Aradi et al. 2015; Siller-Matula et al. 2015).

Increasingly, a deeper phenotyping approach is required to capture the many functions platelets accomplish, through an intrinsically complex array of activation pathways. Comprehensive platelet function testing requires the development of new techniques that allow not only detailed characterization of platelet responses, but are also high-throughput to investigate a large number of individuals. Promising technologies include devices that measure global platelet reactivity in response to shear alone (Wurtz et al. 2012), microfluidic devices with pre-coated adhesion or activation molecules (Conant et al. 2011; Westein et al. 2012; Lucitt et al. 2013; de Witt et al. 2014), assays that measure calcium flux in platelets by fluorescent imaging (Liu and Abell 2006), ELISA-type assays that capture platelets on agonist-coated surfaces (Salles et al. 2010; Baker-Groberg et al. 2014), modified light transmission aggregometry techniques carried out on 96-well plates (Mylotte et al. 2011; Lordkipanidzé et al. 2014) and luminometric assays of platelet secretion in response to various platelet agonists (Sun et al. 2001). All of these techniques have significantly improved our ability to investigate platelet responses to multiple platelet agonists in a shorter time than traditional approaches. In the future, investigators will need to harvest the wealth of data generated by high throughput deep phenotyping approaches and translate these platelet function findings into actionable determinants of disease.

Take Home Messages

- An impressive array of platelet function assays are available to platelet researchers. Many require dedicated skill, expertise, and machinery to run and to interpret, but increasingly, point-of-care instruments are being developed with the intent of applying this technology outside of specialized laboratories.
- Most platelet function assays have strict requirements in terms of blood drawing and sample preparations, in order to help with the standardization and generalizability of results.
- Today's challenges in platelet function testing include determining the true clinical usefulness of these assays for predicting thrombosis and bleeding, and the development of individualized approaches to mitigate risks in individual patients.
- Increasingly, a deeper phenotyping approach is required to capture the many functions platelets accomplish, through an intrinsically complex array of activation pathways. In the future, investigators will need to harvest the wealth of data generated by high throughput deep phenotyping approaches and translate these platelet function findings into actionable determinants of disease.

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Platelet Counting and Measurement of Platelet Dimensions

Patrizia Noris and Carlo Zaninetti

Abstract

Since the introduction of automated blood cell analyzers, platelet counting and platelet dimension measurements are greatly improved in accuracy and disposability. No doubts in considering their relevance evident in routine clinical practice, particularly in diagnosis and management of many bleeding and thrombotic imbalances.

Besides the impedance-based one, the first performed, and to date the most diffused, several others automated counting methods have been developed making platelet recognition increasingly more accurate. Platelet counting is now obtained not only by size but also by density and surface glycoproteins' expression.

Although these technological improvements, spurious abnormalities of platelet count such as pseudothrombocytopenia and pseudothrombocytosis can still occur, posing serious diagnostic difficulties. Therefore, in few selected clinical settings, the manual microscopy techniques are still considered of great utility to correctly identify and enumerate platelets.

Moreover, the widespread automated cell blood counters make today available additional platelet parameters like the mean platelet volume and the amount of young platelets, calculated as reticulated platelets or immature platelet fraction. Although methodological issues remain to be solved, the availability of these further platelets' parameters can also drive effectively the diagnosis of common or rare platelet disorders such as immune thrombocytopenia and inherited thrombocytopenias. The relevance in diagnosis and prognosis of these parameters is still under investigations in a large number of platelet diseases and in disorders not primarily affecting platelets.

Introduction

Platelet counting is essential for the diagnosis and management of many bleeding and thrombotic disorders: its accuracy greatly improved during the last decades with the introduction of automated counters which replaced the routine manual method. Although a number of technical efforts, the impedance analyzers, which are the first-generation automated counters, still cannot distinguish platelets from other particles of the same size because they rely only on particle size. To solve this issue, several counters have been developed based on different techniques in which platelets are recognized on their size and density or on their surface glycoproteins. Despite new methodologies, pseudothrombocytopenia and other spurious abnormalities of platelet count can still occur.

The widespread diffusion of automated counter greatly increased the detection of both inherited and acquired thrombocytopenias and thrombocytosis; emerging age, sex, and ethnical differences of platelet number suggest the need for new personalized reference ranges.

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Automated cell blood counters made available additional platelet parameters: in particular, the diagnostic and therapeutic relevance of young platelets, measured as reticulated platelets or immature platelet fraction, and of platelet size, calculated as mean platelet volume or mean platelet diameter, is emerging although some methodological issue remained to be solved.

Platelet Counting

Platelet counting has become a basic analysis for clinical and research laboratories. In the clinical setting, a reliable enumeration of platelets is required not only for diagnostic purpose but also for therapeutic decision: in fact, clinical guidelines suggest platelet transfusions for platelets less than 10×10^9/L, a number associated with poor reliability of both automated and manual platelet counting. All methods available will be discussed, although only a few of them are routinely used in the clinical practice; they are summarized in Table 1. The main technical characteristics of most automated cell counters now commercially available are reported in Table 2. Methods for enumeration of youngest platelets, namely reticulated platelets, will be discussed with their clinical relevance.

Methods for Platelet Counting

Manual Platelet Counting by Optical Microscopy Since 1988 and until a few years ago, the manual phase-contrast microscopy method, described more than half a century ago, was the only reference technique for platelet counting (Brecher et al. 1953; ICSH 1988): after dilution in ammonium oxalate of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood and lysis of red blood cells (RBCs), platelets are counted in a hemocytometer, by a phase-contrast microscope with a 40× objective, where platelets appear as small and refractive particles (England et al. 1988). Although it is inexpensive and widespread used prior to the advent of automated methods, this technique has a number of significant limitations: it is laborious and time-consuming but especially suffers from imprecision and poor reliability, with an interoperator coefficient of variation of 10–25 %, which is much higher for low platelet counts (Harrison et al. 2000). Nowadays it is used in less-resourced laboratories and in research laboratories. More importantly, it is performed in routine laboratory when platelet count is unexpectedly low and in both routine and research laboratories when atypical platelets are present in the sample (i.e., giant platelets), in order to obtain a more reliable platelet count (Noris et al. 2009; Salignac et al. 2013).

Table 1 Methods for platelet counting

Manual platelet counting	In a hemocytometer, by a phase-contrast microscope
	On a May-Grünwald-Giemsa-stained peripheral blood smear, by an optical microscope
Semiautomated platelet counting	Immunoplatelet counting
Automated platelet counting	Impedance platelet counting
	Optical platelet counting
	Optical fluorescence platelet counting
	Automated immunological platelet counting

Manual Platelet Counting on Peripheral Blood Smear It was developed in the era preceding the introduction of automated counters; later, it was used when automated platelet counting was not reliable because of different reasons (i.e., giant platelets, RBC microcytosis/fragmentation). While the first methods provided indirect platelet counting referred to RBCs (Fonio 1912), a more recent one supplies a platelet count referred to the automated leukocyte count (Sutor et al. 2001). It is now devoted to rule out artifacts leading to spurious increase or decreases in automated platelet counts.

Immunoplatelet Counting Given the poor reliability of the manual phase-contrast platelet counting, the widespread diffusion of automated cell counters, and the number of interferences causing inaccuracy of automated platelet count, an immunological platelet technique has been proposed as the new reference method (Harrison et al. 2000; Harrison et al. 2001); it was soon accepted by the ICSH and the ISLH as the gold standard in the process of whole blood calibration of automated hematology analyzers (ICSH 2001).

It is an indirect method where platelet count is obtained from the ratio of fluorescent platelets to RBCs contained in the same sample. Platelets are labeled with directly conjugated, fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies (MoAbs) against two distinct epitopes of integrin $\alpha_{2b}\beta_3$; sample is then analyzed in a flow cytometer where platelets are definitely identified and distinguished from non-platelet elements based on their fluorescence (Log FL1) and cell granularity (Log FS). A minimum of 50,000 total events and 1000 platelet events are collected to determine the RBC/platelet ratio. Afterward, the reference platelet count is calculated from this ratio and the RBC count obtained in a semiautomated, single-channel, impedance counter using the formula: impedance RBC count/(RBC events/PLT events). In the validation procedure, this method demonstrated an excellent intra-assay and acceptable inter-laboratory precision (ICSH 2001); it is especially useful in

Table 2 Principal automated blood cell counters available for analysis of platelet parameters

Counter, manufacturer	Platelet counting method	Platelet parameters, routine, and research only (*)
ABX Pentra, Horiba	Impedance	PLT, MPV, PDW, PCT
Advia, Siemens	Optical	PLT, MPV, PDW, PCT, MPC, PCDW, L-PLT
BC-6800, Mindray	Impedance, optical fluorescence	PLT-I, PLT-F, MPV, PDW*, P-LCR (%), P-LCC (#), PCT, IPF (% and #)*
CELL-DYN, Abbott	Impedance, optical, immunological	PLT-I, PLT-O, MPV, PDW, RetPLT (%)
DxH, Beckman Coulter	Impedance	PLT, MPV, PDW
XE and XN series, Sysmex	Impedance, optical, optical fluorescence	PLT-I, PLT-O, PLT-F, MPV, P-LCR (%), PCT, IPF (% and #), H-IPF (% and #)*

PLT platelets, *PLT-I* impedance platelet counting, *PLT-O* optical platelet counting, *PLT-F* optical fluorescence platelet counting, *MPV* mean platelet volume, *PDW* platelet distribution width, *RetPLT* reticulated platelets, *PCT* plateletcrit, *P-LCR* platelet large cell ratio, *P-LCC* platelet large cell count, *MPC* mean platelet component, *PCDW* platelet component distribution width, *L-PLT* large platelets, *IPF* immature platelet fraction, *H-IPF* high fluorescence immature platelet fraction, % indicates a percentage, # indicates an absolute number

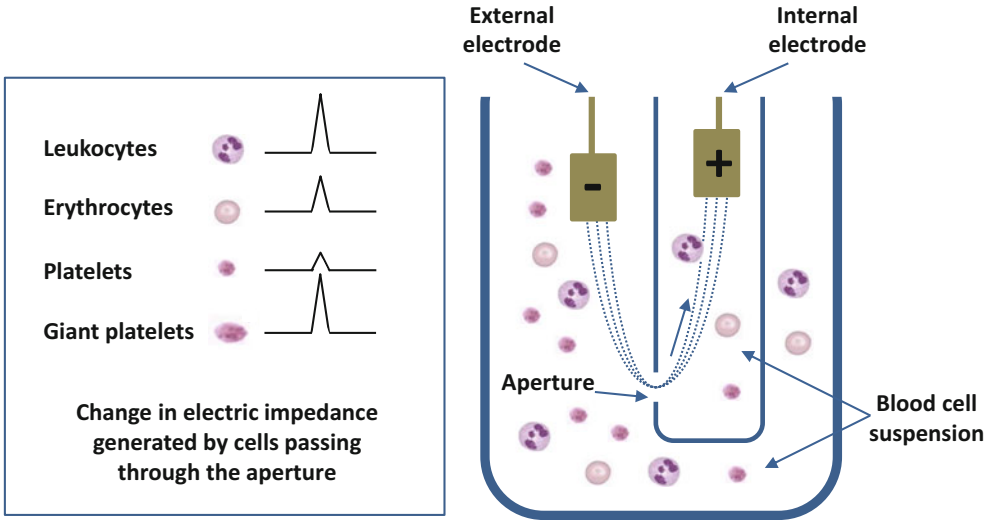

Fig. 1 The principle of impedance platelet counting (Coulter principle). During the analysis, a constant direct current is maintained between two electrodes. When a blood cell passes through a small aperture encompassed by two electrodes (sensing zone), a change in electric impedance is detected: the number of pulses corresponds to the number of particles, the amplitude of the pulse corresponds to the size of individual cells

cases of severe thrombocytopenia, where a reliable platelet count may influence prophylactic platelet transfusion use (Norris et al. 2003). It is not suitable for routine laboratory use because it is time-consuming and quite expensive, but especially because it requires the availability of a flow cytometer and experienced technicians.

Impedance Platelet Counting It is based on the so-called Coulter principle, or impedance method, patented by Coulter in 1953 which made possible the development of the first automated method for cell counting: blood cells are regarded as nonconductive resistivity particles resuspended in a diluent which serves as an electrolyte solution. When a blood cell passes through a small aperture encompassed by two electrodes (sensing zone), a change in electric impedance is detected (Fig. 1): the number of pulses corresponds to the number of particles that pass through, and the amplitude of the pulse corresponds to the size of individual cells. This method, which is the most widely used, was originally used for counting of RBCs and leukocytes in whole blood, and for platelets in platelet rich plasma as at the beginning it was not possible to differentiate RBCs from platelets. It was only in the 1970s that the correction of the coincidence and the introduction of hydrodynamic focusing allowed a reliable enumeration of platelets in whole blood samples (Patterson 1997): the first phenomenon is responsible for incorrect cell counting when more than one single cell passes simultaneously through the sensing zone, and its magnitude increases with the concentration of cells in suspension. The hydrodynamic focusing has been developed to ensure that the stream of cells remains focused in the very center of the orifice of the detecting zone: this prevents the generation of irregular impedance profiles resulting in false estimates of the cell size when they pass through the sensing zone close to the wall where the current density is higher. Further additional improvements have been made to overcome the

recirculation problem leading to an overestimation of platelet count occurring when platelets are trapped in eddy currents behind the orifice where they recirculate and are recounted (Patterson 1997).

The commonly in use impedance analyzers (Table 2) calculate the platelet number by counting blood elements within a specific size range, which is different among the different counters. In the Beckman Coulter DxH800, the platelet size distribution count is produced between two floating windows: one threshold is fixed at 12 fL, while the lower and the upper may move between certain limits in order to distinguish platelets from debris (at the lower end moving between 2 and 6 fL) and from small/fragmented erythrocytes (at the upper end moving from 12 to 25 fL). A platelet size histogram is generated, and afterward, log-normal curves are fitted to this histogram and actual data are calculated. In the LH750 analyzers, particles between two fixed thresholds (2 and 20 fL) are counted as platelets, with possible extrapolation up to 60 fL.

Inaccuracies of platelet counting obtained by methods relying on cell size can result in both overestimation and underestimation of platelet count (Table 3). A false increase of platelet counting can be observed in the presence of non-platelet particles with the same size of platelets, as small or fragmented erythrocytes, fragments of nucleated cells, bacteria, fungi, lipids, and cryoglobulins (Latger-Cannard et al. 2009; Noris and Balduini 2014). Conversely, a false decrease in the platelet number can be observed in case of platelet agglutination (mainly related to EDTA, but also to other anticoagulants), platelet satellitism around leukocytes, platelet-neutrophil agglutination, and in the presence of large platelets exciding the normal range of platelet size (Fig. 1) (Zandecki et al. 2007; Latger-Cannard et al. 2009; Noris et al. 2009). Some abnormalities of platelet count will be further discussed more extensively.

Optical Platelet Counting Optical light scatter methods have been developed more recently, based on light diffraction in a flow cytometry system: when cells are hit by a bundle of light, they can absorb the light, or scatter it, into different directions depending on a number of cellular properties. One-dimensional platelet analysis have been developed first; because the discriminant to distinguish platelets from non-platelets was based only on the size of blood elements, the method did not offer significant advantages over impedance counting. Then, a two-dimensional laser system has been developed (Fig. 2) where platelets are recognized both on their size and granularity. The light scattered by each cell passing through a laser beam is measured at two different angles and converted into volume (platelet size) and refractive index (platelet density). Different counters use different angles: Siemens counters measure two angles of laser diffraction at 2–3° and 5–15°, converted into volume and refractive index, respectively, allowing the recognition of non-platelet particles and large platelets between 1 and 60 fL (Harris et al. 2005). CELL-DYN Sapphire platelet parameters are obtained measuring light scatter at 7° and 90°: in this counter platelets are measured simultaneously by impedance method, and an alert flag is generated when discrepancies are detected suggesting the presence of interfering phenomenon. By optical method, a number of inaccuracies of impedance platelet counting have been overcome: small or fragmented RBCs with the same volume of platelets are recognized because of a greater refractive index, while RBC ghosts are separated from platelets for a reduced refractive index. Moreover, large platelets with volumes between 30 and 60 fL are correctly identified based on their peculiar density: in a study on patients with different inherited macrothrombocytopenias, the optical method was found to be more reliable than the impedance one (Noris et al. 2009).

Table 3 Conditions responsible for spurious platelet count obtained by automated cell counters and possible additional alterations of complete blood count parameters (in bracket)

Spurious decrease of platelet count	Spurious increase of platelet count
Clotting of the sample for inadequate mixing blood sample, or overfilling of the sample tube, or difficult venipuncture (abnormal CBC)	Red blood cells microcytosis (abnormal PLT histogram and RBC distribution width)
In vitro hemolysis (spurious reduction of RBC)	Fragmented red blood cells (abnormal PLT histogram and RBC distribution width)
Venipuncture site, i.e., site proximal to a drip or blood collected from a line (spurious reduction of RBC and WBC)	Cytoplasmic fragments of nucleated blood cells (abnormal RBC distribution width)
Platelet agglutination, i.e., EDTA-dependent pseudothrombocytopenia or related to other anticoagulants (spurious increase of WBC)	Microorganisms, i.e., bacteria and fungi
Platelet satellitism around white blood cells and phagocytosis	Cryoglobulins and cryofibrinogen (spurious increase of WBC and RBC)
EDTA-dependent platelet-neutrophil agglutination (spurious reduction of WBC)	Lipids (spurious increase of WBC and RBC)
Giant or large platelets (abnormal RBC distribution width)	

PLT platelets, *RBC* red blood cells, *WBC* white blood cells

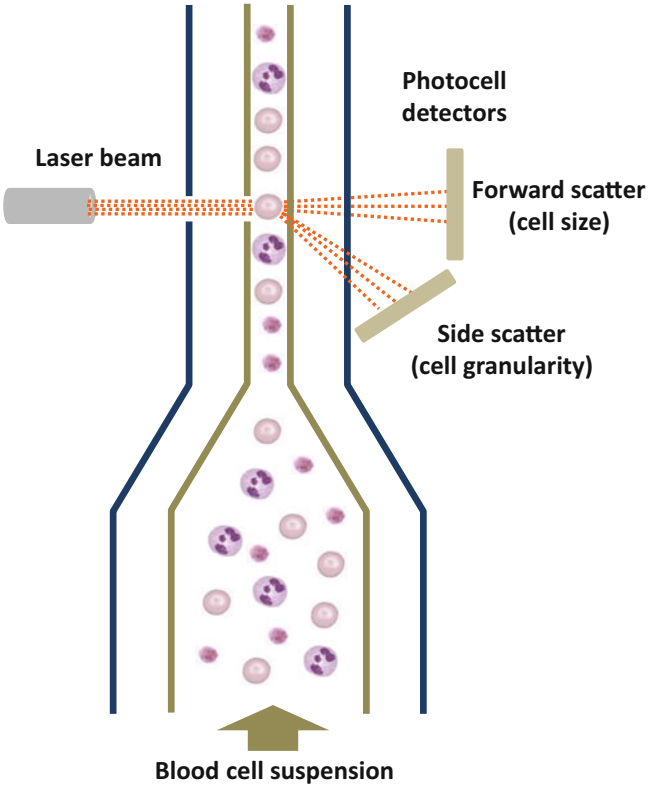

Fig. 2 The principle of optical platelet counting. A cell suspension, passing through a chamber as a single cell stream, interact with a laser beam. The light scattered by each cell is measured at two different angles (forward scatter and side scatter) and generates pulses converted to electronic signals providing information on cell size and internal structure/density

Optical Fluorescence Platelet Counting It is available in two counters (Table 2), the Sysmex XN (starting from Sysmex XE-2100 analyzer) and the Mindray BC-6800, where platelets are counted in the reticulocyte channel. A fluorescent dye is used to stain nucleic acids of reticulocytes and platelets, which after irradiation with a semiconductor laser beam are correctly recognized and counted based on their volume (forward scattered light) and side fluorescence intensity. This technique is present in automated analyzers in addition to impedance method, allowing the recognition of large platelets and the exclusion of non-platelet particles. A switching algorithm has been designed to report the most accurate counting: the optical fluorescence technique resulted more reliable at low platelet count when clinical decisions on platelet transfusions are more critical (Briggs et al. 2012; Schoorl et al. 2013) and in the presence of fragmented RBC (Tanaka et al. 2014). Not conclusive results have been reported for platelet count of patients undergoing chemotherapy, probably because of an erroneous staining of white cell fragments following apoptosis (Van der Meer et al. 2003; Tanaka et al. 2014).

Automated Immunological Platelet Counting A fully automated immunoplatelet count has been initially introduced on CELL-DYN 4000 analyzer and is now available also on the CELL-DYN Sapphire (Table 2). This method relies on the ability to recognize all platelets because they are labeled with a specific FITC MoAb against β_3 integrin. During analysis, the counter aspirates blood inside a special tube containing the lyophilized MoAb fixed to the bottom of the tube. After appropriate incubation, the cell suspension is passed through an optic bench where light scatter is measured at the two angles of 7° and 90° along with the fluorescent emission. The number of CD61+ platelets will be obtained taking into account the dilution of the sample, the dilution flow rate, and the duration of the analysis. This method may be regarded as the fully automated version of the international reference method (ICSH 2001), and the unique which is potentially able to overcome all erroneous platelet counts deriving from non-platelet artifacts (Cid et al. 2010). It is easy to perform, rapid, fully automated, and therefore not requiring skilled technicians; however, its usage is strongly limited in the routine laboratory practice because of the poor diffusion of the specific counter and the cost of each single platelet analysis. It can provide underestimation of platelet count in presence of platelet aggregates, while it is useless in patients with Glanzmann Thrombasthenia.

Reference Interval of Platelet Count

Defining laboratory reference intervals in a healthy population and identifying differences occurring during life, between sexes and within different ethnic groups, are important for both clinical orientations and therapeutic decisions. This also applies to platelets, especially for counts only slightly altered compared to the upper and lower limits of the reference interval: it is especially true now that a lower platelet threshold ($\geq 450 \times 10^9$/L) has been identified as a major criteria for the diagnosis of essential thrombocythemia (Tefferi and Vardiman 2008), a disease associated with increased risk of thrombosis. On the other hand, a slight reduction of platelet count might remain stable over time without developing any disorder (Stasi et al. 2006), meaning that, at least in a selected population, no specific, expensive, and invasive tests are always needed.

The current range of platelet count (150 to 400–450 $\times$ 10^9/L), used in the large majority of Western countries, has been identified based on results obtained from several thousands of blood samples of unselected donors analyzed after the introduction of automated counters (Giles 1981; Graham et al. 1987; Biino et al. 2013). Thanks to the increased diffusion of cell counters, subsequent studies placed

some doubt on their universal validity: in particular, previous small and further large population studies remarked that platelet count may vary by sex (Biino et al. 2013), age (Santimone et al. 2011; Biino et al. 2011; Biino et al. 2013; Ambayya et al. 2014; El Graoui et al. 2014; Qiao et al. 2014; Adeli et al. 2015), ethnicity (Bain 1996; Segal and Moliterno 2006), and also within the same geographic area (Biino et al. 2012; Hong et al. 2015). Therefore, many authors suggested the need for personalized reference ranges for platelet count rather than the adoption of generalized intervals and cutoff. The first proposal for a reference interval of platelet count taking into account gender, age, and ethnicity was made a decade ago in the USA (Cheng et al. 2004): it resulted to be unsuitable for clinical practice and is not currently used. A new personalized reference interval has been recently proposed for the Italian population based on data collected on 40,987 inhabitants of 7 Italian areas including 6 geographic isolates (Biino et al. 2011; Santimone et al. 2011; Biino et al. 2012): estimated reference intervals for platelet count have been provided for three age classes (children, under 15 years; adults, between 15 and 64 years; old, over 64 years) and by sex, limited to adult and old people (Biino et al. 2013). This new range has been recently applied to a consecutive series of 917 adult Italian patients admitted to a single institution: the personalized range reduced by 21 % the number of patients with thrombocytopenia, but more importantly, it reduced by 44.8 % the number of subjects with unexpectedly low platelet count (Zaninetti et al. 2015). Based on this first experience, the introduction of the new range into the clinical practice is expected to prevent many subjects to receive a series of unnecessary and expensive tests, this benefiting both involved people and the health system.

The mechanisms of age- and sex-related differences of platelet count are unknown; it is possible to speculate that sex-related differences, which become more evident after puberty, might be partially related to both hormonal differences and body iron content (Evstatiev et al. 2014).

The molecular basis of platelet number heterogeneity is under active investigations: multiple genetic loci have been found to influence interindividual variation in platelet traits, which might be involved in multiple functional pathways relevant to thrombopoiesis (Soranzo et al. 2009; Gieger et al. 2011; Qayyum et al. 2012; Shameer et al. 2014). Five genes (*ACTN1*, *ETV7*, *GABBR1-MOG*, *MEF2C*, and *ZBTB9-BAK1*), one of which already known to be responsible for an inherited macrothrombocytopenia (Pecci 2016), have been recently identified as genetic factors associated with platelet count in a genome wide association study in Hispanic/Latino Americans (Schick et al. 2016).

Reticulated Platelets and Immature Platelet Fraction

Reticulated platelets (retPLT), described by Ingram and Coopersmith in 1969, are the youngest platelets into the blood stream, named "reticulated" in analogy with reticulocytes: they contain residual amounts of RNA which become evident after supravital staining with methylene blue (Ingram and Coopersmith 1969). More recently, a flow cytometric assay analyzing retPLT based on their RNA thiazole orange staining (Kienast and Schmitz 1990; Richards and Baglin 1995) was developed and used to investigate the clinical utility of retPLT in a large number of conditions, mainly characterized by thrombocytopenia, thrombocytosis, or thrombosis (Hoffmann 2014). Although all studies confirmed that retPLT may represent a useful and noninvasive marker of megakaryopoiesis, they also pointed out a number of critical methodological issues (type and concentration of the dye, incubation time, temperature) only partially related to lack of standardization: the major problem is the non-RNA-specific binding of the dye, resulting in a background of fluorescence dependent on the platelet size, which may be solved only by applying a two-dimensional gating process (Matic et al. 1998). This method, unsuitable for routine clinical application, has been overcome by the introduction of a fully automated technique in two counters (Table 2): in the Sysmex XE and XN, where retPLT were renamed "immature platelet fraction" (IPF), and in CELL-DYN Sapphire by Abbott. In the XN Sysmex, IPF are measured in a dedicated fluorescence platelet assay where platelet RNA is stained with a oxazine-based dye: IPF is derived from the forward light scatter intensity, reflecting cell size, and side fluorescence intensity, reflecting the amount of nucleic acid within the cells. In the Abbott analyzers, retPLT are measured in the reticulocyte assay based on the fluorescence dye CD4K530: platelets are distinguished from erythrocytes by a multi-dimensional separation obtained recording fluorescence against an intermediate angle scatter (7°). Since comparison of IPF counting by Sysmex and retPLT by CELL-Dyn Sapphire showed weak correlation, it has been concluded that they probably reflect a different aspect of thrombopoiesis and cannot be used interchangeably (Meintker et al. 2013). In healthy subjects the reference range for Sysmex IPF is 0.5–6.6 %, while for Abbott retPLT is 0.4–6.0 % (Hoffmann 2014). A correlation between retPLT and age has been described in healthy subjects, while differences among sex were not significant (Hoffmann et al. 2013). This parameter is now also available on a third cell counter, the BC-6800 from Mindray.

Clinical Utility of retPLT and IPF The first application of retPLT or IPF was in the field of differential diagnosis of thrombocytopenias: it is now accepted that retPLT/IPF decrease in case of bone marrow failure and reduced platelet production, while they increase in case of accelerated platelet destruction, such as in immune thrombocytopenia (ITP) (Hoffmann 2014; Adly et al. 2015; Sakuragi et al. 2015). In ITP patients, the IPF correlates with the phase of the disease and the response to treatment: patients with active ITP have the highest IPF, and those in complete remission the lowest (Adly et al. 2015). Searching for tests predictive of bleeding in pediatric ITP patients to make therapeutic decisions more appropriate to the severity of the disease, higher percentages or absolute counts of IPF were associated with higher bleeding scores (Greene et al. 2014; Frelinger et al. 2015) suggesting a role as markers of future bleeding risk in ITP.

RetPLT and IPF are considered a valuable marker for monitoring the thrombocytopenic phase after chemotherapy and the hematopoietic assessment after hematopoietic stem cell transplantation (Zucker et al. 2006; Michur et al. 2008; Martinelli et al. 2009; Goncalo et al. 2011; Hoffmann 2014; Morkis et al. 2015): in both conditions their number generally increases 2–3 days before platelet recovery, and by most they are considered precious tools in the decision for platelet transfusion, better than the platelet count (Hennel et al. 2012). In some cases the absolute count of IPF was a better indicator rather than its percentage for predicting platelet recovery after chemotherapy in pediatric patients (Have et al. 2013).

In the field of acute coronary syndrome (ACS), former studies have associated the rate of IPF with premature coronary artery disease, acute cardiovascular events, and impaired response to antiplatelet agents (Gonzalez-Porras et al. 2010; Perl et al. 2014); more recent studies did not confirm the correlation between IPF and the occurrence of residual high-on treatment platelet reactivity (Verdoia et al. 2015), neither the ability of IPF to assist clinicians in the diagnosis of ACS in patients presenting with acute chest pain (Berny-Lang et al. 2015).

The role of these parameters remains to be better defined also in many other diseases where they are extensively investigated.

Measurement of Platelet Dimensions

Since a long time, there is a general consensus that the evaluation of platelet size may be a useful tool in differential diagnosis of platelet disorders; only more recently this parameter has also been explored in diagnosis and prognosis of a variety of thrombotic and non-thrombotic diseases: the usefulness of this parameter has yet to be fully established because of several potential laboratory issues.

Information on platelet size can be acquired by two different methods: by automated counters (Table 2) which measure the mean platelet volume (MPV) and, manually, by optical microscopy, where the mean platelet diameter (MPD) is obtained on peripheral blood smears.

Mean Platelet Volume

After the introduction of automated counters, the MPV has become a simple, inexpensive, and widely available parameter in clinical practice. However, a number of pre-analytical and intra-analytical factors, summarized in Table 4, may result in unreliable MPV.

Pre-analytical Variables The method of venipuncture and the accuracy of filling the tube and mixing the sample may account for altered MPV since the possible platelet activation occurring in the tube may lead to aggregation of platelets which will not be included in both counting and sizing of platelets (Table 3).

Among all available anticoagulants, EDTA and sodium citrate are the most commonly used in the routine analyses and their effect on MPV the most extensively investigated. Since 1954, platelet exposure to EDTA was known to be associated with change of platelet morphology (Zucker and Borrelli 1954) from elliptical to spherical and with cellular swelling secondary to changes in membrane permeability, dilatation of the open canalicular system, and decreased number of internal organelles (White et al. 1999; White 2000; Diaz-Ricart et al. 2010). Such platelet swelling is responsible for an increased MPV when measured by impedance technology but for a decreased MPV obtained by optical technology, reflecting a reduced platelet density. Different concentrations of a same anticoagulant may result in different effects on platelet volume: a platelet swelling has been described with high (1:4 v/v with blood) but not with low concentrations (1:9 v/v with blood) of sodium citrate (Bath 1993). Based on this methodological problem, in 1993 the ICSH recommended the use of K_2-EDTA (1.5–2.2 mg/mL blood) as reference method:

Table 4 Analytical variables influencing the measurement of mean platelet volume

Pre-analytical variables
Method of venipuncture, accuracy of filling the tube and mixing the sample
Type of anticoagulant
Temperature of storage
Time to analysis
Intra-analytical variables
Platelet counting principle of automated analyzer
Calibration of the automated analyzer

nevertheless, many anticoagulants are still used in routine and research settings (England et al. 1993).

Changes of MPV start immediately after blood exposure to anticoagulant and proceed over time: the MPV increase is usually higher during the first 2 h but it can be recorded also after 24–39 h (Jackson and Carter 1993; Diaz-Ricart et al. 2010; Lancé et al. 2012). In K_2-EDTA blood samples, the MPV may increase up to 27 % after 24 h; the phenomenon may be partially prevented by the addition of inhibitors of signaling pathways and storage at 4 °C (Diaz-Ricart et al. 2010).

Only a few studies explored the influence of the storage temperature of blood samples on MPV: cooling of samples from 37 °C to 4 °C resulted in a reduction of MPV up to 18 % in sodium citrate samples (Threatte et al. 1984; Park et al. 2002). A significant increase in MPV has been recently reported also for EDTA-anticoagulated samples, but only after 24 h storage at room temperature or at 4 °C (Daves et al. 2015).

Intra-analytical Variables By impedance counters, the MVP is calculated on the basis of the volume of elements which are recognized, and counted as platelets based on their dimensions (for details, see "Impedance Platelet Counting"): MPV is derived from the platelet size distribution curve generated during platelet counting by dividing the plateletcrit for the number of platelets (Briggs 2009). Inaccuracies of MPV measurements can potentially result from all conditions leading to under- or overestimation of platelet count (Table 3) but in particular in the presence of giant platelets exciding the normal range of platelet size (Fig. 3) (Zandecki et al. 2007; Noris et al. 2009, Lancé et al. 2012): in patients suffering from *MYH9*-related disease or biallelic Bernard-Soulier syndrome, for example, either the number of platelets or the MPV may be unreliable being underestimated or not even calculated (Noris et al. 2009; Pecci et al. 2014). Moreover, MPV values may differ not only because they are achieved by impedance counters from different manufacturer but also by similar platforms depending on their calibrations (Latger-Cannard et al. 2012; Lippi et al. 2015).

In optical counters the MPV is obtained measuring the light scattered at the lower angle of laser diffraction (for details, see "Optical Platelet Counting"); a platelet histogram is produced from measurements corresponding to the size and number of platelets, and then the MPV is calculated from the geometrical mode of such histogram (Briggs 2009). Based on the specific technology, both platelet counting and sizing obtained by optical counters are expected to be more reliable than the impedance ones: in particular, large platelets are correctly identified based on their peculiar density and then correctly counted and sized.

The reference range of MPV has been investigated in different populations because of the increasing interest about its role in several disorders. Since population studies have been achieved with different cell counters and without standardization of reading times, MPVs of healthy peoples resulted very different, ranging from 6.0 to 13.2 fL in the different studies (Demirin et al. 2011; Hoffmann 2012; Lippi et al. 2012).

No definitive results are available on sex variability: while no differences were observed in a large Italian population study (Santimone et al. 2011), MPV was higher in females compared with males enrolled in a population-based study on adult German individuals (Panova-Noeva et al. 2016). In particular, higher MPV values were found to be significantly associated with age, smoking, hypertension, and glucose levels in men and oral contraceptives and menstruation in women. Importantly, seven and four single nucleotide polymorphisms in women and man, respectively, were found to be significant determinants of larger platelet size (Panova-Noeva et al. 2016).

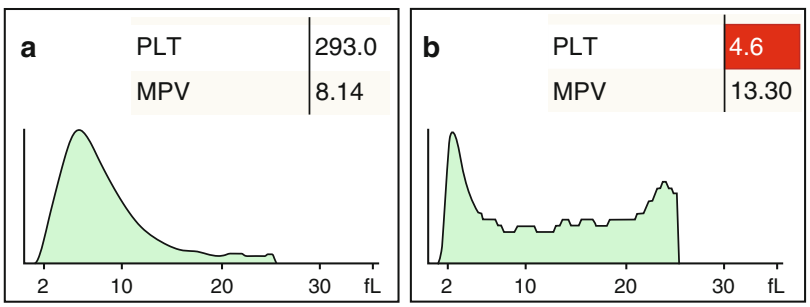

Fig. 3 Histograms of platelet distribution size obtained by the impedance counter DxH800 from Beckman Coulter. Panel **a**: Histogram obtained from a healthy subject (platelets 293 × 10^9/L, MPV 8.14 fL). Panel **b**: Histogram from a *MYH9*-related disease patient with severe thrombocytopenia and extreme platelet macrocytosis. The impedance counter underestimated the MPV of the patient (MPV 13.3 fL, normal range 8–13 fL); because of platelet macrocytosis, also the automated platelet counting was significantly underestimated with respect to the manual counting obtained by optical microscopy (4.6 × 10^9/L vs 32 × 10^9/L, respectively)

Mean Platelet Diameter

An alternative method for platelet sizing has been developed to overcome the abovementioned variables affecting MPV by automated cell counters: it relies on the measurement of platelet diameters by optical microscopy on May-Grünwald-Giemsa-stained peripheral blood films and software-assisted image analysis. The largest diameter of each platelet is measured and the mean platelet diameter (MPD) is the mean value obtained in 200 platelet measurements (Noris et al. 2009) (Fig. 4). Although it is time-consuming and difficult to apply in clinical practice, this method has some strengths: it allows the collection of reliable and comparable measurements in different laboratories and from samples collected long before the analysis. There are no potential variables interfering with the MPD measurement, except for an optimal monolayer preparation of the smear which should be obtained from non-anticoagulated capillary blood. The normal range of MPD has been calculated in 55 Caucasian healthy people (95 % CI 2.4–2.7 μm, mean 2.58 μm) (Noris et al. 2014).

Clinical Utility of MPV and MPD

The field in which platelet size was definitely proven to have a diagnostic role is that of inherited thrombocytopenias (ITs): in fact, the evaluation of platelet size is the first step to distinguish among the different forms of IT whose number is constantly increasing (Noris et al. 2004; Pecci 2016; chapter "Inherited Thrombocytopenias").

The clinical utility of platelet size has been underlined also in the differential diagnosis of ITP from ITs, whose platelet dimensions are frequently overlapping and increased: however, since MPV may be underestimated in presence of giant platelets, only the simultaneous assessment of MPV and MPD demonstrated diagnostic efficacy (Noris et al. 2009; Noris et al. 2013). In a study evaluating MPDs in ITs and ITP, the percentage of platelets larger (40 %) or smaller (10 %) than half an erythrocyte, a parameter easy to obtain by optical microscopy, turned out to be helpful in discriminating ITP from ITs associated with giant platelets or with normal/reduced platelet size, respectively (Noris et al. 2014).

Although there are several methodological limitations (different methodologies and thresholds for MPV, lack of standardization, heterogeneity of the studies, heterogeneity of patients enrolled in the studies) that must be solved (Lancé 2014), the role of MPV has been explored in a number of non-platelet diseases: an increased MPV was significantly associated with risk of myocardial infarction (Chu et al. 2010), venous thromboembolism (Braekkan et al. 2010), a worse prognosis in cancer patients (Kumagai et al. 2015), and cardiovascular events in patients with coronary artery disease (Asher et al. 2014; Sansanayudh et al. 2015); limited to cancer patients, high MPV have been associated with decreased VTE risk (Ferroni et al. 2014) and improved survival (Riedl et al. 2014).

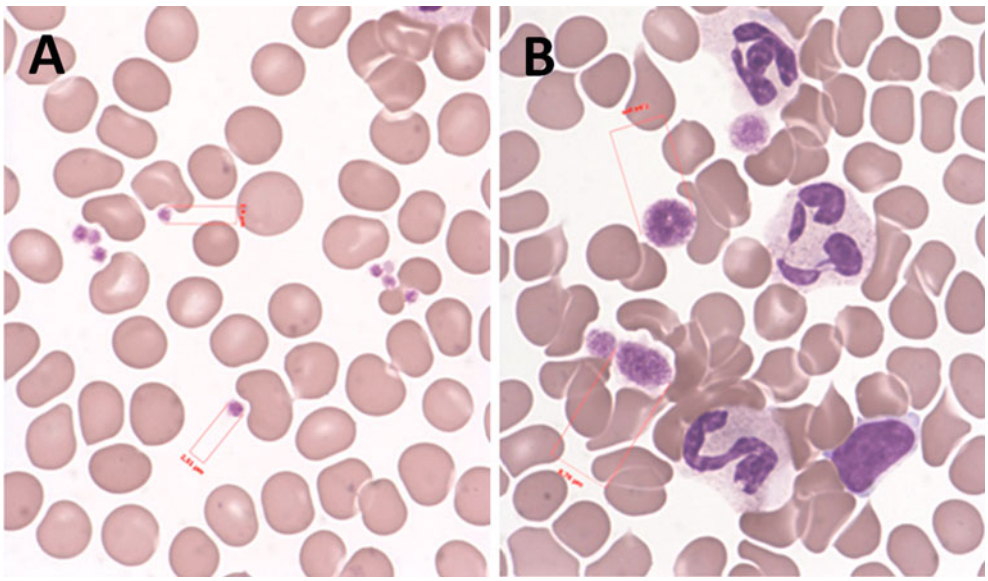

Fig. 4 Measure of platelet diameters provided by the specific tool of the Axio Vision 4.5® software (Carl Zeiss) performed on May--Grünwald-Giemsa-stained peripheral blood smears. Representative examples of the measure are shown. Panel **a**: Measures obtained in a control subject whose mean platelet diameter calculated on 200 platelets was 2.5 μm. Panel **b**: Measures obtained in a patient with *MYH9*-related disease whose mean platelet diameter was 5.6 μm. The range of mean platelet diameter calculated in 55 healthy subjects is 2.2–2.8 μm [Modified with permission from Noris et al. (2009) J Thromb Haemost 7:2131–2136]

In a multivariate analysis combining both clinical and genetic determinants of a large population-based study, Panova-Noeva et al. (2016) observed a higher mortality rate in males with MPV above the upper limit of the reference range during a median follow-up period of 5.0 years.

Finally, the role of MPV as an inflammatory marker still remains to be determined although it is extensively investigated in many different conditions (Varol and Ozaydin 2014; Soydinc et al. 2014; Ataş et al. 2015; Agapakis et al. 2016).

Additional Platelet Parameters from Automated Cell Counters

In addition to retPLT/IPF and MPV, a number of additional platelet parameters may be generated by some automated cell counters (Table 2): among all, the platelet large cell ratio (P-LCR) calculated by ADVIA analyzers represents the number of platelets larger than 12 fL divided by the total number of platelets, while the mean platelet component (MPC) corresponds to platelet density and provides information on anticoagulant induced as well as thrombin-stimulated platelet activation (Salignac et al. 2013). Their clinical utility is still under investigation.

Pseudothrombocytopenia and Other Causes of Spurious Platelet Count

Despite significant technical improvement of automated cell counters, spurious platelet counts can still occur: pseudothrombocytopenia, mainly related to anticoagulants, is the most frequent but a number of other circumstances can lead to erroneous platelet counting (Table 3). They are usually not frequent but their knowledge is important to make practitioners sensible about their possible occurrence, especially in specific clinical setting.

EDTA-Dependent Pseudothrombocytopenia

The frequency of EDTA-dependent pseudothrombocytopenia (EDP) ranges from 0.07 to 0.2 % of all blood counts, and it is not affected by sex but increases with age (Bizzaro 1995; Wu et al. 2011; Xiao et al. 2015). Among patients evaluated for isolated thrombocytopenia, its prevalence is more common in hospitalized than in outpatients (Silvestri et al. 1995; Cohen et al. 2000).

Immunological, chemical, and physical factors are chorally involved in the physiopathology of this phenomenon described first in 1969 (Gowland et al. 1969). It is now acknowledged that platelets' agglutination depends on Abs toward platelet antigens exposed on membrane glycoproteins thanks to EDTA and low temperature action (Onder et al. 1980; Pegels et al. 1982). IgG are more frequently involved, although all Ig classes may elicit the reaction: most of them are cold agglutinins, except for IgM, that usually behave either as room temperature and wide heat range-agglutinins (Onder et al. 1980; Bizzaro 1995). The specific antigen's seat is the $\alpha_{2b}\beta_3$ integrin, especially its α_{2b} subunit (Van Vilet et al. 1986). In basic condition, the epitope is hidden within the integrin. Thanks to the chelating effect of EDTA on calcium ions and low temperature influence, a dissociation of the structure occurs enabling binding of antiplatelet Abs, which in turn cause agglutination (Fig. 5, panel a) (Pidard et al. 1986). Other anticoagulant agents such as heparin, hirudin, oxalate, and citrate may also induce pseudothrombocytopenia (Robier et al. 2010; Nagler et al. 2014; Lin et al. 2015); rarely it can be both EDTA and citrate dependent (<0.02 % of all blood counts) or even more than two anticoagulant dependent (Bizzaro 1995; Zhou et al. 2011). When elicited by EDTA, it could be prevented and sometime reverted by addition of aminoglycosides (Sakurai et al. 1997; Lin et al. 2015). Despite no definite association has been proven, EDP has been frequently observed after viral infections including rubella, mumps, cytomegalovirus,

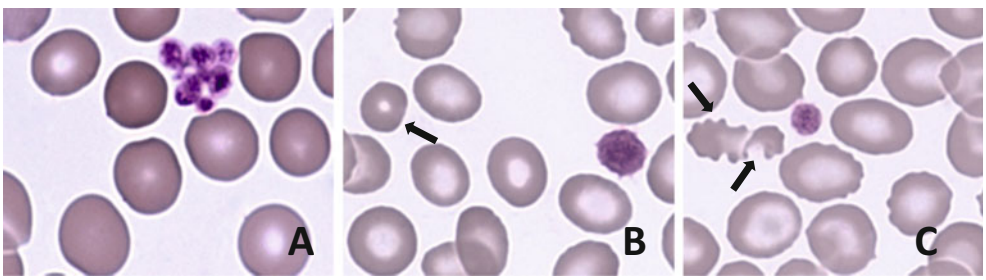

Fig. 5 Conditions leading to spurious low platelet count. Panel **a**: May-Grünwald-Giemsa-stained peripheral blood smear obtained by EDTA-anticoagulated blood from a patient with EDTA-dependent pseudothrombocytopenia: small clumps may be counted as leukocytes leading to pseudoleukocytosis. Panels **b**, **c**: May-Grünwald-Giemsa-stained peripheral blood smear obtained by a patient with β-thalassemia minor and mild thrombocytopenia, due to chronic ITP, where platelets have similar size to some microcytic erythrocytes (*arrow*). In this patient the platelet count was overestimated because microcytic erythrocytes were counted as platelets

Epstein-Barr virus, and hepatitis A virus (Hsieh et al. 2003; Choe et al. 2013).

Clinical Relapses Being a laboratory artifact, EDP is a clinically harmless condition without hemorrhagic risk. Nevertheless, its clinical importance is not irrelevant. A prompt recognition of EDP is mandatory to avoid inappropriate and potentially dangerous treatments or diagnostic procedures: the lack of recognition of EDP in critically ill patients can wrongly influence the process of differential diagnosis (Lau et al. 2004; Kocum et al. 2008; Yoshikawa et al. 2006). Conversely, in a real thrombocytopenic patient for any cause, EDP may overestimate the severity of thrombocytopenia leading to unnecessary platelet transfusion and additional testing (Podda et al. 2012).

Laboratory Picture In case of EDP the electronic cell counters typically reveal a spurious thrombocytopenia because platelet agglutinates are usually counted as small leukocytes. Although the majority of the modern blood analyzers may recognize platelet clumps due to specific scattergram patterns and warning flags, false negatives or positives are not uncommon (Batels et al. 1997): small clumps can be counted as leukocytes and none warning produced except for pseudoleukocytosis (Lombarts and De Kieviet 1988). When a suspicion of EDP is generated, pseudothrombocytopenia can be straightforwardly confirmed by the microscopic observation of platelet clumps in the blood smear obtained by EDTA-anticoagulated tube (Fig. 5, panel a), a procedure useful also for confirmation or exclusion of other causes of machine-determined or overestimated thrombocytopenia, as what happens in all cases of platelet macrocytosis (myeloproliferative syndromes, myelodysplastic disorders, ITs) (Bernard and Soulier 1948; Seri et al. 2003; Noris et al. 2009, Pecci et al. 2014). In these subjects an accurate platelet count may be achieved with alternative anticoagulants like citrate-pyridoxalphosphate-tris (CPT) or magnesium sulfate, effective either for prevention of pseudothrombocytopenia and for obtaining accurate routine hematology data (Lippi and Plebani 2012; Schuff-Werner et al. 2013). When only EDTA-tubes are available, EDP may be avoided collecting blood in a pre-warmed tube and processing the sample, stored at 37 °C, as soon as possible; in alternative, blood may be collected by finger stick and platelets counted manually by optical microscopy (Bizzaro 1995).

Other Causes of Spurious Platelet Count

Platelet Satellitism Named alternatively rosetting or satellitosis, platelet satellitism is a quite rare (1 of 12,000 blood counts) in vitro phenomenon characterized by platelet adherence to normal or abnormal leukocytes in EDTA-anticoagulated blood samples (Zandecki et al. 2007). Polymorphonuclear neutrophils (PMN) are more frequently involved, but rosetting around monocytes, basophils, eosinophils, lymphocytes, and lymphoma cells has been also described (Salignac et al. 2013). Besides EDTA, platelet satellitism has been observed in blood samples anticoagulated with heparin. It has been reported to be unrelated to any pathological conditions, except for some rare reports speculating on a possible relation with autoimmune processes. The responsible mechanism seems to be similar to that of EDP: IgG or IgM auto-Abs against a α_{2b} antigen, unmasked by EDTA, would be accountable for forming bridges between platelets and PMN via their Fcγ receptor (Lazo-Langner et al. 2002).

EDTA-Dependent Platelet-Neutrophil Agglutination The formation of large clumps containing hundreds of PMN and platelets, leading to a spurious reduction of platelet and/or PMN automatic count, could represent the final step of a process started by platelet rosetting around PMN. Because of the low number of cases described, the understanding of mechanism of clumping and the difference with classical satellitism is far to be fully understood (Zandecki et al. 2007). This in vitro phenomenon must be distinguished from platelets-WBC aggregates observed in vivo during thrombotic and inflammatory conditions with marked platelet activation and consequent enhanced expression of P-selectin (Hu et al. 2003).

Platelet Macrocytosis As already discussed (see "Impedance Platelet Counting"), platelet macrocytosis can result in an erroneous low platelet count when obtained by impedance counters relying only on the cell size (Noris et al. 2009; Latger-Cannard et al. 2012).

RBC Microcytosis and Cytoplasmic Fragments of Blood Cells RBC of extremely low volume, such as what occurs in severe iron-deficiency anemia or thalassemia, can disturb the ability of automatic analyzers to distinguish platelets and RBC (Figs. 5, panels b, c and 6) leading to a spurious elevated platelet count and/or reduced RBC count (Savage and Hoffman 1985, Noris and Balduini 2014) since small RBCs are wrongly counted as platelets. Similar eventualities can occur in presence of a large number of schistocytes or microspherocytes, the last during acute burns (Zandecki et al. 2007). Fragments of cytoplasm from other blood cells such as myeloblasts, lymphoblasts, and cells of lymphocytic lymphoma in leukemic phase can lead to a same spurious altered platelet count (Frotscher et al. 2015). Onco-hematologic clinical setting and various types of flags generated by the newest automatic analyzers can suggest the

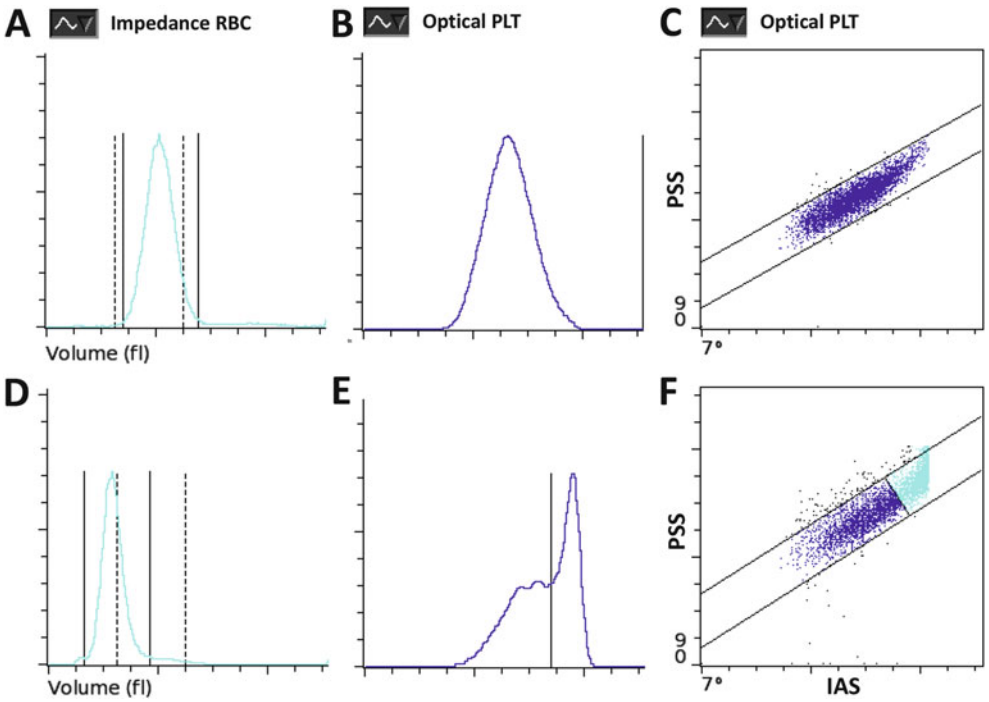

Fig. 6 Impedance red blood cell (RBC) histograms and optical platelet histograms and scatterplots with dynamic threshold produced by a CELL-DYN Sapphire from a normal subject (panels **a**–**c**) and a subject with β-thalassemia minor and mild thrombocytopenia (panels **d**–**f**, see also Fig. 5, panels **b** and **c**). The impedance RBC histogram from the patient clearly displays the red blood cell microcytosis (**d**) interfering with the correct determination of the volume (**e**) and, thereafter, of platelet counting. Microcytic RBCs are visible in the platelet optical scatterplot (**f**, *light blue*) where they are distinguished from platelets (**f**, *dark blue*) based on their density. In this patient the impedance platelet count was 168×10^9/L, while the optical platelet count, based on both dimension and complexity of cells, was 85×10^9/L, superimposable to counting obtained manually by optical microscopy. *IAS* intermediate angle scatter measuring cell complexity/internal structure, *PSS* polarized side scatter measuring nuclear segmentation/lobularity

situations described above that should be ruled out by careful examination of peripheral blood smears.

Microorganisms Spuriously elevated platelet counts may rarely be caused by bacteria or bacterial aggregates, fungi, and trophozoites. These phenomena has been described typically in patients with extremely severe clinical conditions and bacteremia. Similar conditions can even depend on pre-analytical variable such as unsterile tubes used for blood sampling allowing bacterial overgrowth (Zandecki et al. 2007).

Cryoglobulins According to their size, immune complexes precipitating at temperature lower than 37 °C can lead to overestimation of platelet, leukocyte, and rarely RBC count according to their size (Emori et al. 1973). A cryoglobulin-depending spuriously high platelet count typically recovers when repeating the analysis after warming the sample for 30 min at 37 °C. A spurious increased platelet count may be similarly generated by cryofibrinogen (Zandecki et al. 2007).

Lipids Abnormally high levels of lipids in blood (hyperchylomicronemia, samples obtained during parenteral nutrition or infusion of therapeutic agents containing lipids) may result in the in vitro formation of micellae or droplets that can be misdetected as platelets by either optical and impedance automated blood counters (Kabutomori et al. 1999). Similarly to platelets, also hemoglobin concentration and RBC and WBC counts may be affected by abnormal concentrations of lipids (Zandecki et al. 2007).

Take Home Messages

The coming of automated blood cell counters has made platelets' number and dimension evaluation a basic analysis for clinical and research aims. Recently it has been suggested the need for personalized reference ranges for platelets count taking into account gender, age, and ethnicity.

The most commonly in use analyzers, which calculate blood cell number relying on their size, can results in both overestimation or underestimation of platelet count. To solve this matter, several automated methods based on alternative techniques, primarily optical and immunological, have been developed.

(continued)

Despite technical improvements, some methodological issues remain to be solved; in few selected diagnostic routes, the manual method of analysis of platelet parameters (by optical or phase-contrast microscope) is still to be considered irreplaceable.

In clinical setting, platelet count is required not only for diagnostic purpose but also for arduous therapeutic decision. To this aim, having available additional platelet parameters (i.e., large platelets, reticulated platelets, immature platelet fraction) can drive clinical judgment in challenging situations.

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Flow Cytometry

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Abstract

Flow cytometric platelet analysis is becoming increasingly popular for both experimental and clinical applications. Flow cytometry (FCM) can feasibly analyze platelet biology both in vivo and ex vivo, providing information on platelet turnover and count, structure, antigen expression, activation state, interaction with other blood components, and response to agonists.

Reduced sample volume, minimal manipulation, and single-shot multiparametric characterization of platelet populations are the main advantages of FCM platelet studies. However, some aspects need to be carefully considered, including activation-dependent changes resulting from inaccurate sample collection, the need for a dedicated operator, and standardization.

Relevant clinical applications include assessment of thrombotic risk in cardiovascular diseases and cancer, monitoring of pharmacological anti-aggregation, and diagnosis of inherited and acquired platelet function disorders. This chapter focuses on the main applications of FCM in studies that are relevant for both research and clinical settings.

Generalities on Flow Cytometry

First developed as a rapid and automatic technique for determining the nuclear DNA content, flow cytometry (FCM) now finds its most common applications in the phenotypic and functional characterization of various cell types because of its ability to detect several specific characteristics of a large number of individual cells in suspension in a time range of seconds.

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In the flow cytometer, suspended aligned cells pass through a flow cell where they are hit by the focused beam of a laser. Detectors process the light scattering and, in the case of fluorescent labeling, the emitted fluorescence of each cell. Cell parameters that can be assessed by FCM are divided into two groups:

- *Physical or intrinsic parameters*, represented by the forward-scattered light (FS), which is proportional to cell dimensions, and the side-scattered light (SS), which is proportional to the structural complexity of the cell.
- *Fluorescent parameters*, obtained by labeling cells, before analysis, with fluorophore-conjugated monoclonal antibodies (targeting specific cell antigens) or cell-permeable fluorescent proteins that are activated at specific excitation wavelengths by a laser beam. The intensity of the fluorophore-emitted light is directly proportional to the antigen density. The wide range of labeled antibodies or cell-permeable fluorescent proteins commercially available permits not only the analysis of

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several cell parameters in one single shot, but also a detailed study of cell phenotype and function, cell–cell interaction, cell activation, apoptosis, and cell cycle, to name just a few (McCoy 2002).

Advantages of the Use of FCM in Platelet Studies

FCM allows rapid analysis of platelet turnover and count, morphology, structure, activation state, and response to agonists. This means that it is a versatile and reliable diagnostic tool for analysis of platelet disorders, evaluating the effects of antiplatelet agents, and assessment of circulating activated platelets in the context of thrombotic risk.

In recent years, the use of FMC in the field of hemostasis and thrombosis has become widespread thanks to its feasibility compared with time-consuming and expensive conventional platelet assays such as enzyme-linked immunoassay (ELISA), transmission electron microscopy (TEM), fluorescence microscopy, C^{14} labeling, high performance liquid chromatography (HPLC), particle gel immunoassay (PaGIA), aggregometry, and radioimmunoassay (Carubbi et al. 2014a).

Additionally, the reduced blood volume required for analysis, minimal sample manipulation, and high number of commercially available monoclonal antibodies allowing single-shot multiparametric evaluation make FCM a fundamental technique in platelet studies. However, some aspects need to be carefully considered when using FCM in platelet studies. In particular, the operator should be aware of the risk of platelet activation as a result of inadequate sample collection and/or time delays. The principal advantages and disadvantages of FCM are summarized in Table 1.

Methodological Aspects

Peripheral Blood Draw: Suggested Procedure and Sample Preparation

In platelet studies, a careful execution of peripheral blood draw is mandatory to minimize platelet activation. For this purpose, a 20–21G needle is recommended. Additionally, it is helpful to rapidly remove the tourniquet and discard the first 2 mL of blood. The sample should then be conserved at room temperature and processed within 30 min. These precautions are strongly recommended when the activation state of circulating platelets is to be determined.

The choice of anticoagulant relies mainly on specific tests: in the case of ex vivo platelet response to agonists, sodium citrate is recommended because it does not interfere with platelet activation. By contrast, if seeking to minimize platelet activation after sampling, anticoagulants should be chosen that contain platelet inhibitors such as CTAD (citrate, theophylline, adenosine, and dipyridamole). Ethylenediamine tetraacetic acid (EDTA) is suitable for platelet count measurements (Michelson et al. 2000).

Platelet Source

The starting material for platelet studies by FCM can consist of platelet-rich plasma (PRP), washed platelets, or whole blood. The choice depends mainly on the test type. PRP, obtained by blood centrifugation at 125–150 *g* for 10–20 min, is suggested by some authors for calcium flux analysis.For detection of platelet microparticles, two centrifugations (1500 *g* for 15 min followed by 13,000 *g* for 2 min) are recommended in standardized protocols. Washed platelets are utilized in activation studies with strong agonists (i.e., thrombin), which can cause clot formation in the presence of plasma fibrin (Hickerson and Bode 2002; Robert et al. 2009).

Nevertheless, in clinical studies, whole blood is by far the most convenient platelet source (Shattil et al. 1987) because platelets are kept in their physiological milieu of red cells, leukocytes, and plasma components, which clearly affects platelet activation (Santos et al. 1991; LaRosa et al. 1994b). Moreover, the sample is minimally manipulated, preventing artifactual ex vivo activation and potential loss of platelet subpopulations (Michelson et al. 1991; Abrams and Shattil 1991; Shattil et al. 1987). Also, only minuscule volumes of blood are required (2–5 μL), allowing accurate analyses on newborns and profound thrombocythopenic patients (Shattil et al. 1987; Michelson et al. 1991; Rajasekhar et al. 1994). Michelson and colleagues (Michelson et al. 1991; Michelson 1994; Kestin et al. 1993) developed a method for studying

Table 1 FCM in platelet studies

Advantages	Disadvantages
Minimal sample manipulation	Dedicated operator
Minimal volumes required (2–5 μL per test)	Activation-dependent changes as a result of inaccurate sample collection
Physiological milieu (whole blood)	
Applicable in profound thrombocythopenias	
Detection of activation-dependent changes	
Quantitative results	

platelet activation by thrombin directly in whole blood, based on addition of the synthetic tetrapeptide GPRP (glycyl-L-prolyl-L-arginyl-L-proline) that competitively prevents fibrin polymerization and, via inhibition of fibrinogen binding to its receptor, partially blocks platelet aggregation. The same effect is also mediated by Arg-Gly-Asp (RGD)-containing peptides that, mimicking fibrinogen binding sites, act as glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists. These assays allow simultaneous analysis of the activation state and reactivity of circulating platelets. An alternative is use of the thrombin receptor agonist peptide (TRAP), although it may not fully reflect all aspects of thrombin-induced activation because it is a peptide fragment of the "tethered ligand" receptor for thrombin (Yamamoto et al. 1991).

Expression of Antibody Binding

In FCM, platelet population is conventionally identified by a "morphological gate" according to platelet physical properties and is represented on a dot plot of FS versus logarithmic SS. However, for discrimination of platelets from other blood cells it is more appropraite to use a light scatter parameter versus the fluorescence signal of platelet-specific constitutive antigens (i.e., "immunological gate"). Photomultiplier voltages are adjusted to give light scatter signals in the midrange of the logarithmic scale of the instrument. Amplification of fluorescence signal is selected to give a platelet autofluorescence signal that falls within the first logarithmic decade of the instrument. Because of spectral emission overlap, appropriate electronic color compensation must be set for each combination of antibodies (fluorophores) according to the manufacturer's instructions and confirmed by each laboratory.

Antibody binding can be expressed as the percentage of cells staining positive for a particular antibody or as mean fluorescence intensity (MFI). The percentage of positive cells identifies a subpopulation of platelets expressing a specific antigen, in comparison with the negative fluorescence of a predominant platelet population. A threshold value for the positive signal intensity is defined on the basis of an appropriate value for false-positive events, conventionally including 1–2 % of positive cells in a one-parameter histogram analysis of a matched negative control sample stained for the determination of nonspecific fluorescence (e.g., sample stained with isotype control). This method is not affected by variations in signal amplification because the negative control signal increases in proportion with the test sample. Moreover, as a result of its high sensitivity, the method allows detection of changes in antigen expression by small subpopulations of cells, which results in a heterogeneous platelet staining pattern. Indeed, the "percentage of positive platelets" method can detect subpopulations of platelets arising from a local in vivo insult and is the most appropriate method for analysis of antigens expressed upon activation. However, this method is inappropriate for measuring variations in the expression of antigens that are homogeneously exposed across platelet subpopulations (i.e., CD41). Moreover, antibody-positive platelets may have very little antigen expressed at their surface.

If the goal is to determine variations in homogeneously expressed antigens or the total amount of platelet surface antigens, MFI is the method of choice. MFI is defined as the mean fluorescence intensity of the analyzed population and represents the mean antigen density on the cell surface. Therefore, an increase or decrease in MFI represents a similar increase or decrease in antigen expression per single platelet. Quantification of the number of antibodies specifically bound to platelets is based on a calibration curve established for each directly conjugated antibody using multiple bead populations with a defined number of specific binding sites. For this purpose, commercial kits equipped with a set of calibrated fluorescent standards and software can be used to determine molecules of equivalent soluble fluorochrome (MESF). An additional advantage of the routine utilization of these standards allows data comparison over time and between different instruments and laboratories (Michelson et al. 2000).

An interesting technique for the calculation of binding index that takes into account both MFI and the percentage of positive platelets has also been described (Hjemdahl et al. 1994; Zeller et al. 1999; Leytin et al. 2000).

FCM Evaluation of Platelet Activation Markers

As previously stated, FCM is a helpful tool for assessing platelet activation state both in vivo (circulating platelets) and ex vivo (platelet reactivity to agonists). When activated, platelets undergo several changes in surface antigen expression and granule release (Fig. 1). FCM can detect these events as changes in the binding of specific monoclonal antibodies that recognize markers of platelet activation. The most common markers are summarized in Fig. 1 and Table 2 and can be divided into surface markers and intracellular markers. Additionally, other parameters of platelet activation are represented by platelet–leukocyte aggregates (PLAs) and platelet microparticles (PMPs).

Activation-Dependent Surface Markers

Compared with other cells, platelet surface antigens constitute a larger proportion of platelet cellular mass and are primarily represented by platelet–membrane receptors.

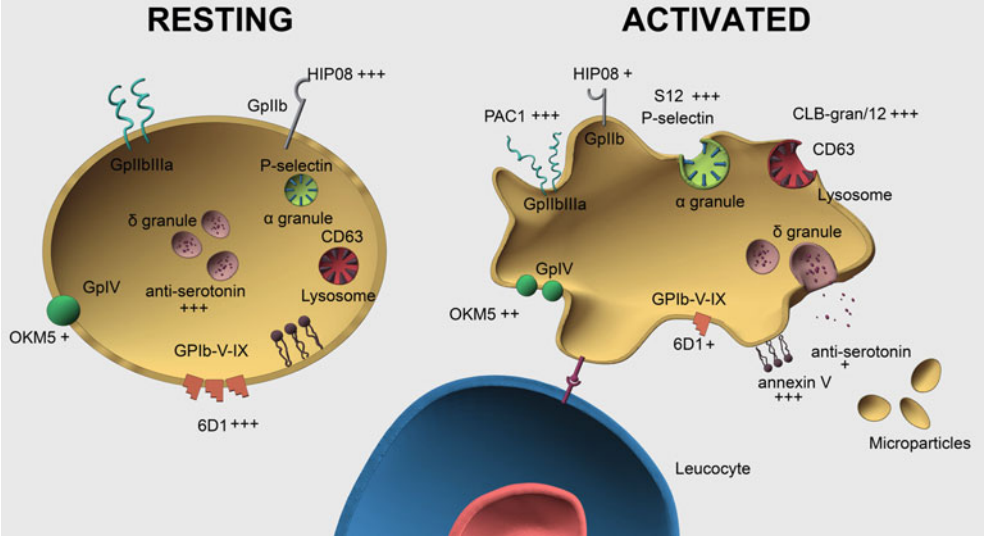

Fig. 1 Surface and intracellular platelet markers in resting and activated platelets studied by flow cytometry

Table 2 Flow cytometric markers of platelet activation

Marker	Description/function	Prototypic antibody	Binding: resting platelets	Binding: activated platelets
Surface markers				
Activated form of $\alpha_{IIb}\beta_3$	Platelet membrane glycoprotein; binds to fibrinogen and vWF	Anti-CD41/CD61 (activated form)	–	+++
P-selectin	Component of α-granule membrane; mediates adhesion to neutrophils and monocytes	Anti-CD62P	–	+++
Lysosomal integral membrane protein	Integral protein of platelet lysosomal membrane and δ-granules	Anti-CD63	–	+++
GPIV	Platelet membrane glycoprotein; binds to collagen and thrombospondin	Anti-CD36	+	++
GPIb-V-IX complex	Platelet membrane glycoprotein; binds to vWF	Anti-CD42a-d	+++	+
Phosphatidylserine	Anionic phospholipid of platelet membrane bilayer	Fluorescently conjugated annexin V	–	+++
Activated form of GPIIb	Platelet membrane glycoprotein complexed with GPIIIa; binds to fibrinogen and vWF	Anti-CD41a, HIP08 clone	+++	+
Intracellular markers				
Serotonin	Stored in δ-granules; mild platelet agonist	Anti-serotonin	+++	+
Vasodilator-stimulated phosphoprotein (VASP)	Intracellular signaling molecule that is not phosphorylated in the basal state and phosphorylated in PGE1-inhibited platelets		+++ (PGE1-inhibited)	–
Others				
Leukocyte–platelet aggregates	Activated platelet–monocyte/neutrophil complexes	Anti-CD41, -CD61, -CD42, -CD3, -CD19, -CD56, -CD16, -CD14	+	+++
Platelet microparticles	Platelet fragments	Anti-CD62P CD107A, CD63	+	+++

Conformational modifications or changes in the levels of their expression on the cellular surface can be used to trace platelet activation and, for this purpose, FCM is the method of choice for its accuracy and rapidity. Platelet–membrane receptors are grouped into eight types:

1. Integrins ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$)
2. Leucine-rich repeat receptors (GPIb-IX-V complex, Toll-like receptors)
3. Seven-transmembrane receptors (thrombin, prostaglandin, ADP, lipid, and chemokine receptors)

4. Immunoglobulin superfamily (GPVI, FcγRIIa, FcεRI, junction adhesion molecules, intracellular adhesion molecules, PECAM-1, CD47)
5. C-type lectin receptor family (P-selectin, CD72, CD93)
6. Tetraspanins (CD9, CD63, CD82, CD515), glycosylphoshatidylinositol-anchored proteins (CD55 and CD59)
7. Tyrosine-kinase receptors (thrombopoietin, insulin and leptin receptor, PDGF receptor)
8. Others (CD36, lysosomal-associated membrane proteins, CD40L) (Clemetson and Clemetson 2013).

The two most widely utilized activation-dependent surface antigens are P-selectin and the $\alpha_{IIb}\beta_3$ complex (Shattil et al. 1985; Stenberg et al. 1985) (see Table 2 and Fig. 1).

P-selectin (or CD62P) is a component of the α-granule membrane of resting platelets that mediates adhesion to neutrophils and monocytes. It is expressed on their surface only after platelet degranulation (Fig. 1). Therefore, P-selectin-specific monoclonal antibodies (see Fig. 2 and Table 2) bind exclusively to activated platelets.

The $\alpha_{IIb}\beta_3$ complex is a receptor for fibrinogen, von Willebrand factor (vWF), vitronectin, and fibronectin. It undergoes a conformational change in response to platelet activation. The change is recognized by the monoclonal antibody PAC-1 (Fig. 1), which binds specifically to the fibrinogen binding site of $\alpha_{IIb}\beta_3$ that is exposed during its activation-dependent re-shaping (see Fig. 2 and Table 2).

As an alternative to $\alpha_{IIb}\beta_3$-specific monoclonal antibodies, fluorescein isothiocyanate (FITC)-conjugated fibrinogen can also be used to detect the activated form of the surface glycoprotein. Fluorescein–fibrinogen binding is saturable, dependent on agonist activation, but competitively inhibited by unlabeled fibrinogen in the plasma and released from platelet α-granules (Faraday et al. 1994; Heilmann et al. 1994).

Other common markers of platelet activation are represented by CD63 (also known as lysosomal integral membrane protein, LIMP), CD36 (or GPIV), and CD40L. CD63 is a component of lysosomal membranes whose role has yet to be clarified. When granules are released, CD63 is exposed on the platelet surface where its expression increases from 650 to 12,600 molecules per platelet (Fig. 1) (Nieuwenhuis et al. 1987). Although CD63, compared with P-selectin, can be considered a more stable marker of platelet activation because of a reduced tendency to undergo proteolysis, it is not an ideal candidate for platelet activation studies because of a requirement for a greater level of activation for adequate surface antigen expression. Consequently, P-selectin remains the marker of choice for the majority of investigations (Nishibori et al. 1993).

The monoclonal antibody OKM5 is directed against an epitope on GPIV that binds thrombospondin. OKM5 also recognizes resting platelets but its binding is increased by thrombin stimulation (see Fig. 1). The GPIb-IX complex binds to vWF, mediating adhesion to damaged blood vessel walls and is recognized by the monoclonal antibody 6D1. Platelet activation leads to a redistribution of GPIb-IX complexes from the surface into the open canalicular system (Hourdille et al. 1990, 1992; Michelson et al. 1994). The decreased binding of 6D1 to activated platelets is a very sensitive marker of activation both ex vivo and in vivo (Kestin et al. 1993) (Fig. 1).

CD40 ligand (CD40L, also known as CD154) is a trimeric transmembrane protein and member of the tumor necrosis factor family. It is a component of the α-granule membrane of resting platelets and is quickly exposed on the platelet surface after activation. Expression of CD40L on the activated platelet surface is transient because it is rapidly cleaved from the platelet membrane, generating a soluble fragment known as soluble CD40L (Aloui et al. 2014). FCM studies with the monoclonal antibody TRAP-1 demonstrated that CD40L is undetectable on resting platelets and that platelet activation by thrombin results in maximal expression of CD40L within 1 min (Henn et al. 1998). Platelet activation in cardiovascular disease is associated with a significant increase in expression of CD40L (Garlichs et al. 2001; Abu el-Makrem et al. 2009; Pignatelli et al. 2011; Ferroni et al. 2012). However, in the majority of recent papers, detection of soluble CD40L by ELISA is preferred to detection of surface CD40L, probably because of the transient expression of the latter (Ferroni et al. 2012).

Binding of FITC-conjugated annexin V is a widely utilized marker of platelet activation. A crucial step in the activation of the coagulation cascade is the result of a flip–flop in anionic phospholipids (predominantly phosphatidylserine; PS) from the inner to the outer leaflet of the platelet membrane bilayer, which forms a binding substrate for the prothrombinase complex (Fig. 1). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein that allows detection of PS exposure on the outer cell surface of activated platelets (Dachary-Prigent et al. 1993) (see Fig. 2 and Table 2).

Intracellular Markers

FCM can be utilized to evaluate intracellular markers such as specific δ-granule components (serotonin and adenine nucleotides) and specific phosphoproteins. Serotonin release from the δ-granules, where it is stored in steady-state conditions, is a crucial step for platelet aggregation (Fig. 1). However, its measurement has relied for years on expensive and laborious techniques (HPLC, ELISA, C^{14} labeling). Our group has described an FCM method for assessing intracellular serotonin content in resting and activated platelets after fixation and permeabilization using

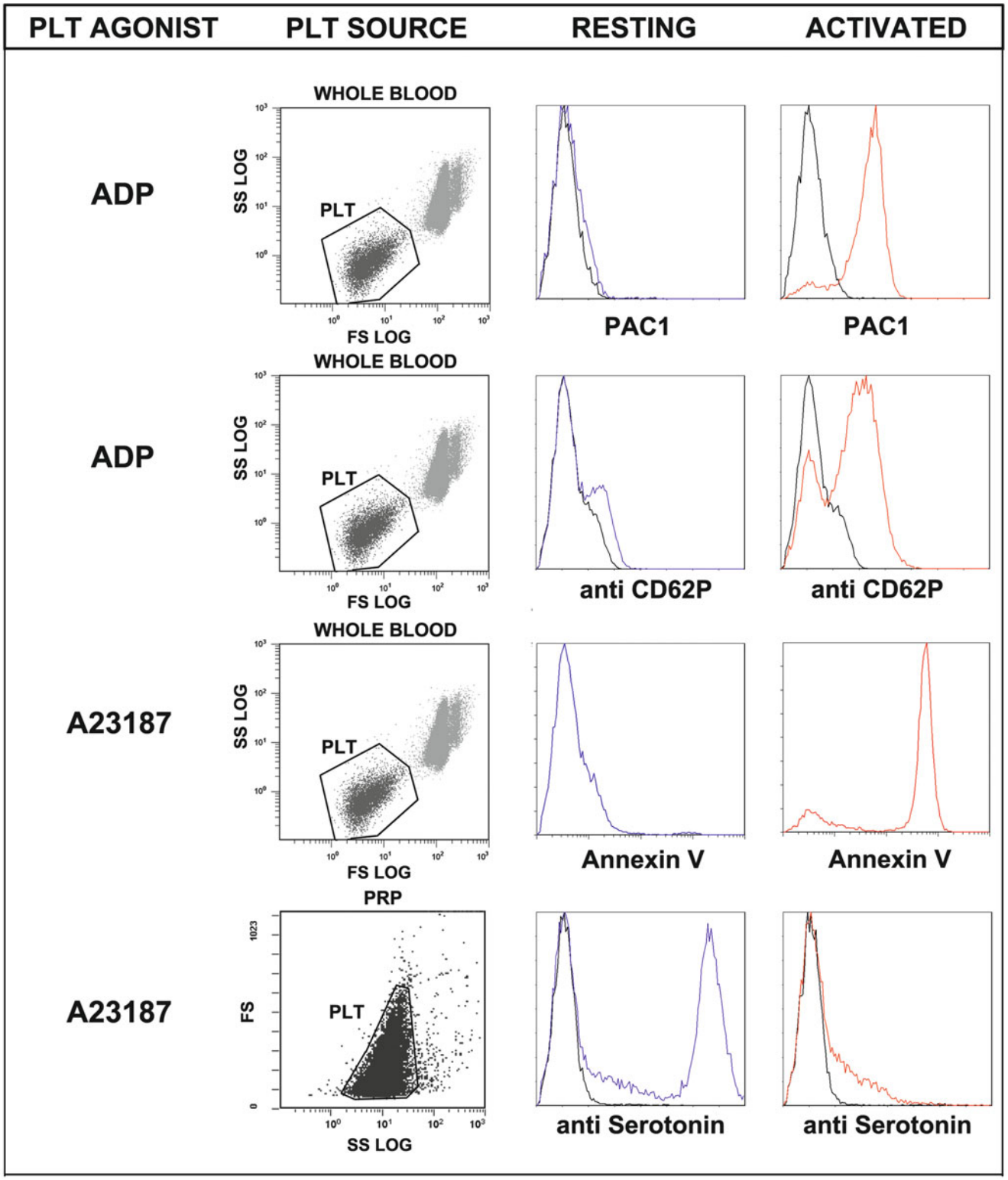

Fig. 2 Flow cytometric analysis of the most utilized activation markers before and after platelet activation with different agonists (ADP and calcium ionophore A23187). Analysis was performed on whole blood or platelet-rich plasma (PRP), as indicated

an anti-serotonin–R-phycoerythrin (RPE) conjugated antibody coupled with anti-CD41 surface marker staining (Gobbi et al. 2003) (see Fig. 2 and Table 2).

Adenine nucleotides can be detected by mepacrine (quinacrine) staining. Mepacrine is a green fluorescent dye capable of selectively binding adenine nucleotides and, consequently, provides information on the uptake and release of δ-granule content (Gordon et al. 1995).

Vasodilator-stimulated phosphoproteins (VASPs) are intracellular signaling molecules that are nonphosphorylated in the basal state and phosphorylated in prostaglandin E1-inhibited platelets. VASPs are targets of the ADP/$P2Y_{12}$ receptor, a seven-transmembrane domain receptor linked to an inhibitory G-protein. The degree of VASP phosphorylation (reported as platelet reactivity index) reflects $P2Y_{12}$ activity and can be measured using a commercial assay developed by BioCytex (Schwarz et al. 1999).

Platelet–Leukocyte Aggregates

As previously described in chapter "Platelet-Leukocyte Interactions" (Evangelista et al. 2017), it has been well known since 1989 that activated platelets bind to monocytes or neutrophils via P-selectin interaction with the PSGL-1 counter-receptor on the leukocyte surface (Larsen et al. 1989). It has been demonstrated that the level of circulating platelet–leukocytes aggregates (PLAs) is increased in pathological conditions such as ischemic stroke (Marquardt et al. 2009) and diabetes mellitus (Elalamy et al. 2008) and in patients with coronary artery disease (Mickelson et al. 1996; Furman et al. 1998; Ott et al. 1996; Neumann et al. 1997b; Sarma et al. 2002). PLAs are currently utilized as platelet activation markers (Nagasawa et al. 2013; Pearson et al. 2009) (Fig. 1 and Table 2).

FCM is a sensitive and rapid method for qualitative (mean fluorescence intensity) and quantitative (percentage positive events) measurement of PLAs. To minimize ex vivo platelet activation attributable to centrifugation and washing steps, whole blood analysis is recommended. In addition, sample fixation reduces uncontrolled changes in the platelet surface. Although some authors discourage red cell lysis because it can lead to artifactual activation (Pearson et al. 2009; Li et al. 1997), other investigators have used red cell lysis to provide clearer delineation of platelet and white cell populations, reporting that the lysis procedure does not affect platelet–monocyte aggregation (Furman et al. 1998; Ray et al. 2005; Harding et al. 2007).

Usually, FCM detection of PLAs is based on a combination of a platelet-specific antibody (usually CD41, CD42 and/or CD61) and leukocyte-specific antibody. Specifically, in whole blood analysis, leukocytes can be discriminated from erythrocytes by anti-CD45 binding. Moreover, the specific leukocyte population interacting with platelets can be identified using antibody against CD3 (T-lymphocytes), CD19 (B-lymphocytes), CD56 (natural killer cells), CD14 (monocytes), CD16 (neutrophil), anti-CD4, and anti-CD8 (to discriminate T cell subsets) (Michelson et al. 2001; Yip et al. 2013; Pearson et al. 2009; Nkambule et al. 2015) (Table 2).

Platelet-Derived Microparticles

As previously mentioned in chapter "Platelet-Derived Microparticles" (Cointe et al. 2017), platelet microparticles (PMPs) are fragments with a size ranging from 0.1 to 1 μm. They circulate in the bloodstream at a concentration of 100–1000/μL. They are shed from platelets when activated and during aging and destruction; therefore, they have a phospholipid-based structure with functional receptors belonging to the platelet membrane. Specifically, PMPs express common megakaryocyte-platelet glycoprotein receptors (CD41, CD42b) and platelet activation markers such as PS, P-selectin, and CD63. In recent years, there has been increasing interest in PMPs because of their clinical applications. PMPs are used as platelet activation markers ex vivo, such as after stimulation with specific agonists such as thrombin, collagen, and calcium ionophore A23187 (Michelson et al. 2001; Italiano et al. 2010) (Fig. 1 and Table 2), and also in vivo. In fact, aberrant levels of plasma PMPs have been associated with bleeding or thromboembolic complications and can be considered a biomarker of ongoing thrombosis (Ramacciotti et al. 2009).

PMP detection techniques have been implemented and new methodologies are currently under investigation, including TEM, ELISA, FCM, atomic force microscopy, nanoparticle tracking analysis, and resistive pulse sensing (Nomura et al. 2009; Dragovic et al. 2011; Yuana et al. 2010). At present, FCM is still the most widely utilized method for PMP identification.

Several methodological issues must be taken into account when analyzing PMPs from peripheral blood. PMP detection can be affected by several sample processing steps, ranging from blood collection, plasma isolation, and storage to staining of phospholipids and surface antigens for determining the cellular origin of the microparticles (Shah et al. 2008; Shet 2008).

Although the standardization of pre-analytical steps remains a challenge, an overall consensus has been reached on the following specific issues (Lacroix et al. 2010, 2012; Yuana et al. 2011): It is recommended that samples are collected carefully to avoid sheer stress and endothelial activation, using a light tourniquet, large needles (20–21G), and discarding the first 2–3 mL of blood. Citrate tubes are preferred over EDTA, because the latter is known to interfere with microparticle measurement (Shah et al. 2008; Trummer et al. 2009). In a hospital setting, it is

important to note that patients' samples are not collected in the laboratory, therefore sample transportation and time delay between blood sampling and microparticle preparation can impact the analysis. It was demonstrated that a time delay of 2 h before the first sample centrifugation is acceptable because any increase in the number of microparticles during this period remains moderate. Moreover, transporting blood tubes in the vertical rather than horizontal position limits the extent of in vitro microparticle generation (Lacroix et al. 2012).

Platelets need to be removed from the plasma to avoid cellular activation, which can lead to inadvertent production of microparticles. For this purpose, the most common protocol for FCM microparticle analysis consists of one centrifugation at 1500 *g* for 15 min followed by a second centrifugation at 13,000 *g* × 2 min to obtain platelet-free plasma (Yuana et al. 2011; Robert et al. 2009; Sabatier et al. 2002). However, some investigators perform the analysis directly on platelet-poor plasma obtained after a first blood centrifugation at 1500 *g* for 15 min and a second re-centrifugation of plasma at 1500 *g* for10 min (Jy et al. 2004; Enjeti et al. 2008; Dignat-George et al. 2009).

Lacroix and coworkers demonstrated that two successive centrifugations of 2500 *g* for 15 min at room temperature are more efficient for platelet removal (Lacroix et al. 2012). Dey-Hazra and colleagues also suggested that the filtration of buffer using a 0.2-μm filter is a useful step for reducing the amount of background noise, cell debris, and precipitates. The latter have the same size range as microparticles and could influence or disturb the analysis (Dey-Hazra et al. 2010).

In multicenter studies and prospective trials it is often inevitable that plasma samples are frozen and stored before performing the assay. In these cases, plasma is first snap-frozen in liquid nitrogen before being stored at −80 °C, or, alternatively, plasma is directly frozen at −80 °C (Yuana et al. 2011). It has been demonstrated that directly freezing at −80 °C and quickly thawing in a water bath at 37 °C at the time of the assay do not strongly influence PMP analysis (Lacroix et al. 2012).

Regardless of the biological source (peripheral blood or cell culture), one of the most important steps for PMP detection is the identification of PMP populations according to their light scattering properties. Indeed, PMPs are submicrometer fragments, therefore their discrimination from platelets and debris relies on the setting and resolution of the instruments used.

To promote standardization, the ISTH SSC Working Group on Vascular Biology proposed the use of calibrated latex beads with sizes of 0.5, 0.9, and 3 μm (MegaMix Beads BioCytex, Marseille, France) to adjust the instrument settings and increase the resolution of FCM. The dimensions of this bead population cover both the PMP (0.5 and 0.9 μm) and platelet populations (0.9 and 3 μm) (Lacroix et al. 2010; Robert et al. 2009). A multicenter study, performed with several types of FCM, demonstrated that standardization of PMP enumeration by FCM is feasible but dependent on the intrinsic characteristics of FCM and on the calibration strategy. Although latex beads remain an imperfect model for defining the cutoff of PMP populations, because the refractive index of the plastic beads is different to that of PMPs, they are currently considered useful standards that allow instrument qualification and follow up (Lacroix et al. 2010).

PMP staining with fluorescently labeled antibodies is recommended for more precise identification of PMP populations. PS is a well-established marker for PMPs, regardless of their cellular origin. Fluorophore-labeled annexin V is commonly used to measure the total number of PMPs with FCM. However, more specific platelet antigens should be used to distinguish PMPs from microparticles derived from leukocytes, erythrocytes, and endothelial cells. Von Willebrand factor and fibrinogen receptors are markers for microparticles derived from both platelets and megakaryocytes; however, monoclonal antibodies specific for platelet activation markers such as P-selectin, CD107A, and CD63 are recommended for selective identification of PMPs (Flaumenhaft et al. 2009).

To provide the concentration or absolute count of PMPs in a sample, counting beads of a known concentration are used. When they are used as internal standard, the beads are added to each sample before FCM measurement. For use as an external standard, the counting beads are processed at the same FCM settings/conditions used for the samples. FCM counts the number of beads and/or microparticles in the sample until the acquisition time is reached and, from this number, the concentration of microparticles can be calculated (Yuana et al. 2010; van der Zee et al. 2006; Robert et al. 2009; Shet et al. 2003).

Ex Vivo Platelet Activation

Platelet reactivity after ex vivo stimulation with different agonists can be performed on whole blood, PRP, or washed platelets (Fig. 2). Whole blood analysis allows detection of high degrees of platelet activation, limiting selective cell losses (Shattil et al. 1987).

Several agonists, both fully synthetic or isolated from organisms, are commercially available and allow highly reproducible and standardized ex vivo activation. Such agonists include ADP, stable thromboxane analogs, and PAR1 agonists such as TRAP-6 (SFLLRN) and TRAP-14 (SFLLRNPNDKYEPF) (Giesberts et al. 1995; Michelson et al. 1991). GPVI-specific agonists include collagen-related peptide [Gly-Lys (or Cys)-Hyp-(Gly-Pro-Hyp)10-

Gly-Lys (or Cys)-Hyp-Gly] (Morton et al. 1995; Kehrel et al. 1998), the snake toxin convulxin (Polgar et al. 1997), and poly(Pro-Hyp-Gly), which induces platelet aggregation independently of thromboxane A_2 and $\alpha_2\beta_1$ (Inoue et al. 2009).

Shear stressed-induced activation can also be used: the friction forces generated by forcing the sample through a capillary tube produces a platelet activation state similar to that registered in vivo at an injury site or stenotic vessel. The activation markers that can be monitored are listed in Table 2.

Main applications of ex vivo platelet activation studies are monitoring of antiplatelet drugs and pathological conditions associated with platelet hypo- or hyperreactivity, as described next.

In Vivo Platelet Activation

An activated platelet phenotype may reflect an ongoing acute thrombotic process such as acute coronary syndrome, as well as chronic pathological conditions such as diabetes, peripheral arteriopathy, allergic asthma, or cancer (Tschoepe et al. 1991; Tomer 2004). Analysis of circulating activated platelets (i.e., in vivo platelet activation) requires extremely careful sample collection and manipulation to avoid activation induced by venipuncture and storage. If the sample cannot be processed within a short time frame, stabilization with activation antagonists and fixatives is strongly encouraged (Shattil et al. 1987; Dovlatova et al. 2014). Platelet subpopulations that are heterogeneous in their activation state can be detected; in fact, the lower limit of detection of platelet activation for FCM assays is about 1 % activated platelets in a sampled population of stimulated and unstimulated platelets (Shattil et al. 1987).

FCM is the most appropriate technique for in vivo platelet activation studies. Basically, all the FCM platelet activation markers previously described can be utilized to assess the activation state of circulating platelets (Table 2), although some concerns have been raised concerning the transient expression of P-selectin and the rapid reversibility of activated $\alpha_{IIb}\beta_3$ (Schmitz et al. 1998). The choice of marker relies mainly on the clinical application and the disease of interest. Platelet degranulation markers such as P-selectin have been utilized in hypertension (Stumpf et al. 2005) and activated $\alpha_{IIb}\beta_3$ in antiphospholipid antibody syndrome (Joseph et al. 2001). PLAs and PMPs have been extensively studied in solid and hematologic malignancies, acute myocardial infarction, coronary revascularization procedures, diabetes, and asthma (Michelson et al. 2001; Mallat et al. 2000; Pitchford et al. 2003; Falanga et al. 2005).

Platelet Turnover

Platelet Count

Obtaining accurate platelet counts has been a long-standing problem for both pathologists and clinicians, as demonstrated by the fact that platelet counting methods have evolved from manual phase contrast microscopy through the era of automated cell analyzers to FCM, which is currently considered the gold standard technique, as discussed in detail in chapter "Platelet Counting and Measurement of Platelet Dimension" (Noris and Zaninetti 2017).

In FCM, platelets are identified with a fluorescent monoclonal antibody specific to a cluster of differentiation common to all (resting and activated) platelets, with the advantage that platelets are immunologically recognized independently of size. Thus, FCM allows unequivocal identification of platelets from other cellular elements of similar size. This is of great relevance in thrombocytopenic samples, for which platelet counting is known to be problematic because of the presence of giant platelets or particles (i.e., fragmented red cells and immune complexes). In these cases, FCM platelet count (in contrast to impedance analysis or optical counting) can include giant platelets in the count because they are clearly resolved from red cells, and particles with sizes similar to those of platelets are excluded (Ault et al. 1999; Tanaka et al. 1996; Harrison et al. 2001).

Because most flow cytometers cannot be programmed to process a fixed volume of sample, counting procedures involve indirect derivation of platelet number. For this purpose, various approaches have been proposed (Tanaka et al. 1996; Davis and Bigelow 1999). Specifically, in the late 1990s, an immunoplatelet counting procedure was introduced that was based on a reference standard of fluorescent beads with a predefined concentration. In this method, a known amount of fluorescent calibration beads is added to the sample, and platelet number calculated as bead ratio (Dickerhoff and Von Ruecker 1995; Matzdorff et al. 1998). However, this method lacks standardization and an alternative procedure using FCM platelet count based on red blood cells as internal standard has been demonstrated to satisfy the criteria for a reference method (Harrison et al. 2001).

The International Council for Standardization in Haematology (ICSH) and the International Society of Laboratory Hematology (ISLH) recommend FCM-based counting, based on the ratio of platelets to red blood cells, as a reference method for platelet counting (International Society of Laboratory Hematology Task Force on Platelet Counting, International Council for Standardization in Haematology Expert Panel on Cytometry 2001).

The assay involves dilution of EDTA-anticoagulated blood specimen in a sterile buffered solution, followed by staining of platelets with specific fluorescent antibodies. The

stained platelets in solution are diluted to the counting concentration, and the platelets and red blood cells (RBCs) counted on a flow cytometer with thresholds set to discriminate platelets from RBCs on the basis of fluorescence amplitude and scatter amplitude. The RBC/platelet ratio is determined, and the platelet count is calculated from an accurate RBC count of the sample, obtained using a cell counter that meets previous ICSH specifications (International Council for Standardization in Haematology 1994). The main advantage of this technique is that, by using RBCs as internal standard, the count obtained is independent of potential pipetting artifacts (Harrison et al. 2001).

Multicenter studies using different approaches have been undertaken to improve the accuracy of platelet counts (De la Salle et al. 2012; Masters and Harrison 2014; Sehgal et al. 2010; Sandhaus et al. 2002; van der Meer et al. 2012).

Reticulated Platelets

Reticulated platelets (or young platelets) are rich in mRNA. Platelet mRNA derives from megakaryocytes during thrombopoiesis, and, because of its instability, is present in a larger amount in young platelets than in older ones and can be utilized to identify newly released platelets from the bone marrow.

FCM is the method of choice for reticulated platelets analysis. This technique relies on the use of a fluorescent dye, thiazole orange (TO), which has a large fluorescence enhancement and high quantum yield upon binding to nucleic acids, especially RNA. Reticulated platelets can thus be distinguished from mature platelets by FCM according to their dye uptake (Kienast and Schmitz 1990; Bonan et al. 1993).

Protocols have been successively optimized to associate TO labeling with platelet-identifying antibodies for better characterization of platelet populations (Chavda et al. 1996) and a consistent number of papers describing clinical applications of reticulated platelet analysis have been published. However, it also became evident that the FCM assay is prone to methodological variation, which makes it difficult to compare results obtained with different assays; for example, the normal reference range is reported to lie between 1 and 15 % (Romp et al. 1994; Matic et al. 1998). Many factors that contribute to this analytical issue have been identified: type and concentration of fluorescent dye, incubation time and temperature, fixation, RNAse treatment, and FCM data analysis, including gating and threshold settings (Richards and Baglin 1995; Watanabe et al. 1995; Matic et al. 1998; Bonan et al. 1993; Rapi et al. 1998). One of the major problems is that platelets show non-RNA-specific binding of fluorescent dye, resulting in background staining, which is size-dependent (Matic et al. 1998; Robinson et al. 1998; Balduini et al. 1999). New initiatives have been undertaken that are aimed at developing a method with the potential to become a future international reference method (Hedley and Keeney 2013; Machin 2013). According to these studies, reticulated platelet analysis should be performed in whole blood, with stringent control of incubation time and temperatures and with sample fixation after labeling. Concerning gating strategy, platelets should be identified according to CD41 and CD61 expression and then analyzed using a dot-plot of SS versus TO. A threshold of 1 % for TO-positive platelets is defined using a negative control unstained for TO. Platelet enumeration is performed according to the ICSH guidelines using the platelet/RBC ratio as described above (Hedley et al. 2015; Hoffmann 2014).

The main clinical application of reticulated platelet assessment is to determine, in thrombocythopenic patients, whether the low platelet count is a result of abnormally accelerated destruction or of impaired bone marrow output. Because megakaryopoietic activity is low in patients with bone marrow failure, the assumption is that the ratio of reticulated platelets to total platelets is also low. By contrast, in conditions typified by enhanced compensatory megakaryocypoiesis (e.g., immune thrombocytopenia or postchemotherapy recovery) the absolute number of reticulated platelets, and consequently the ratio of reticulated platelets to total platelets, is expected to be high (Kienast and Schmitz 1990; Pons et al. 2010; Thomas-Kaskel et al. 2007; Rinder et al. 1998; Ryningen et al. 2006; Romp et al. 1994; Richards et al. 1996; Catani et al. 1999; Stohlawetz et al. 1999). Interestingly, in the thrombocytopenic phase after chemotherapy and transplantation for hematological malignancies, reticulated platelets have been monitored by FCM and it has been observed that an increase in reticulated platelets precedes the recovery of platelet count by 2–3 days. This creates the opportunity to defer platelet transfusions that would be given if transfusion decisions were based only on platelet count. However, clinical evidence supporting this concept is limited and there is need for randomized, controlled clinical studies in this field (Macchi et al. 2002; Wang et al. 2002; Michur et al. 2008; Chaoui et al. 2005).

Reticulated platelets have also been utilized for risk assessment and drug monitoring in patients with coronary artery disease (Guthikonda et al. 2008; Lakkis et al. 2004; McBane et al. 2014; Perl et al. 2014).

Recently, Hedley and colleagues (Hedley et al. 2015) demonstrated that macrothrombocytopenic patients show an increase in the immature platelet fraction that, in agglutination tests, increases during the formation of platelet clumps. The data indicate that measurement of the immature platelet fraction is influenced by platelet size and could be a useful parameter in the differential diagnosis of macrothrombocytopenia (Miyazaki et al. 2015; Hoffmann 2014).

Clinical Applications

Clinical Disorders Associated with Abnormal Platelet Reactivity

Several thrombotic and nonthrombotic disorders are typified by alterations in the number of circulating activated platelets and an abnormal expression of platelet activation markers. Both these conditions can be evaluated by FCM.

Coronary Syndromes

FCM studies have demonstrated the presence of circulating activated platelets in patients affected by coronary artery disease (CAD), including stable angina, unstable angina, and acute myocardial infarction (AMI), as well as after percutaneous coronary intervention (PCI). In this setting, analysis of platelet activation markers is not only beneficial in terms of diagnosis but also provides information on clinical outcome and can be utilized for the optimization of antiplatelet therapies

Specifically, CD62P-expressing platelets and circulating PLAs at baseline and after stimulation with exogenous agonists are higher in patients with stable CAD, unstable angina, and AMI than in healthy subjects (Furman et al. 1998; Langford et al. 1996; Carubbi et al. 2012).

Interestingly, these two platelet activation markers, both detected by FCM, show a different sensitivity. PLAs are a more sensitive marker of in vivo platelet activation than CD62P in patients that have undergone PCI and with AMI, and are therefore potential candidates as early diagnostic markers of AMI (Michelson et al. 2001; Furman et al. 2001).

Other studies reported an increase in PMPs, CD40L, and platelet collagen receptor (GPVI) in acute coronary syndrome (Katopodis et al. 1997; Bigalke et al. 2007; Ferroni et al. 2012; Skeppholm et al. 2012).

Interestingly, analysis of the expression of platelet activation markers can be of prognostic value for thrombotic risk after PCI. It has been demonstrated that patients developing acute events after PCI showed a higher expression of platelet CD62P, CD63, and the active form of $\alpha_{IIb}\beta_3$ before intervention. The authors suggest that FCM analysis of platelet function in PCI candidates can stratify patients into those at high and low risk of cardiac events after PCI (Kabbani et al. 2001, 2003; Tschoepe et al. 1993; Gawaz et al. 1997).

Cerebrovascular Ischemia

It has been extensively demonstrated that almost all platelet activation markers, including CD62P, CD63, activated $\alpha_{IIb}\beta_3$, PMPs, and PLAs, are increased in patients with cerebrovascular ischemia (McCabe et al. 2004; Cao et al. 2009; Grau et al. 1998; Zeller et al. 1999; Meiklejohn et al. 2001; Yamazaki et al. 2001; Cherian et al. 2003; Yip et al. 2004; Smout et al. 2009; Tsai et al. 2009; Marquardt et al. 2002; Htun et al. 2006; Minamino et al. 1998; Bigalke et al. 2010; Lee et al. 1993; Geiser et al. 1998; Fateh-Moghadam et al. 2005; Koyama et al. 2003). Interestingly, the expression levels of these markers have been associated with different subtypes of ischemic stroke.

Specifically, patients with large-vessel cerebral infarction elicit higher platelet activation than those with small-vessel infarction, whereas the role of platelet activation is still controversial in cardioembolic stroke (Cao et al. 2009; Grau et al. 1998; Zeller et al. 1999; Meiklejohn et al. 2001; Yamazaki et al. 2001; Cherian et al. 2003; Yip et al. 2004; McCabe et al. 2004; Smout et al. 2009; Tsai et al. 2009; Marquardt et al. 2002; Oberheiden et al. 2012; Turgut et al. 2011). Furthermore, FCM analysis of CD62P, CD63, and PMPs suggests that platelet activation occurs chronically in these diseases, as demonstrated by the fact that, despite the specific effects of antithrombotic therapies, their expression levels are higher at 1, 3, and 6 months before the acute event than in healthy controls (Grau et al. 1998; Cherian et al. 2003; Meiklejohn et al. 2001; Yamazaki et al. 2001; Marquardt et al. 2009).

Aggregation with different leukocyte subtypes was also investigated. Results showed that an increase in monocyte–platelet aggregates is short-lived and could reflect an acute reaction to cerebral ischemia. By contrast, granulocyte–platelet aggregate formation persists into the subacute phase, suggesting that specific PLAs could reflect the prothrombotic state and the inflammatory processes after stroke (Marquardt et al. 2009).

Peripheral Vascular Disease

The use of FCM to assess platelet function in patients with peripheral arterial disease (PAD) has been documented since 1991. Data published in the last 10–15 years show an increase in platelet hyperreactivity and circulating activated platelets in these patients compared with healthy subjects. It has been demonstrated that P-selectin expression, the number of PMPs, and the number of platelet aggregates are significantly higher for both resting and stimulated platelets in the PAD group compared with controls (Robless et al. 2003; Cassar et al. 2003; Koksch et al. 2001; Zeiger et al. 2000). Interestingly, platelet activation parameters are correlated with the severity of vascular disease. P-selectin and PLAs, specifically platelet–monocyte aggregates (PMAs), are significantly increased in patients with severe limb ischemia compared with those with intermittent claudication (Rajagopalan et al. 2007; Tan et al. 2005). Moreover, it has been demonstrated that in the early postoperative period after infrainguinal bypass, the level of PLAs, including PMAs and platelet–neutrophil aggregates, is

significantly greater in patients who had experienced graft occlusion compared with patients whose graft remained patent at 6 months. The authors suggest that this analysis allows identification of a patient subset with a high risk of graft occlusion that would benefit from more aggressive antiplatelet therapy (Esposito et al. 2003).

All patients affected by peripheral venous disease, irrespective of the degree of chronic venous insufficiency, are characterized by higher levels of PMAs and platelet–neutrophil aggregates in the bloodstream and an increased propensity to form platelet aggregates in response to platelet agonists than control group subjects. However, this increase in platelet reactivity appears unrelated to the presence of ulceration (Powell et al. 1999). Moreover, higher circulating levels of PMAs persist after complete correction of chronic venous insufficiency, suggesting that the increased number of PMAs identified in these patients is not secondary to the presence of venous reflux, but could be involved in the primary etiology of chronic venous insufficiency (Rohrer et al. 2002).

Cancer

The association between platelet and cancer dates to the mid-1800s, when Armand Trousseau linked venous thrombus formation with an underlying undiagnosed malignancy (Dammacco et al. 2013). Since then, several studies have reported platelets as major players, not only in thrombus generation in neoplastic patients, but also in promoting tumor growth, invasiveness, and angiogenesis. Many authors now point to the "platelet–cancer loop" as a pivotal mechanism in tumorigenesis (Hasselbalch 2014). In this setting, detection of platelet activation markers by FCM is as a useful tool. For solid tumors, most reports concern tumor-derived microparticles. Toth and colleagues showed that PMPs are higher in breast cancer patients than in those with benign lesions. PMP levels correlate with tumor invasiveness, but no association was established with prothrombin levels and thrombin formation (Toth et al. 2008). In hormone-refractory prostate cancer, Helley and coworkers demonstrated a correlation between circulating PMP levels and Gleason score, patient performance status, and overall survival, with a poorer outcome in patients with more than 6867 PMPs/μL (Helley et al. 2009). In patients with gastric cancer, the mean number of CD62P molecules on the platelet surface was significantly higher than in the healthy group, and further increased after stimulation with TRAP (6- to 12-fold in neoplastic patients and threefold in the control group) (Osada et al. 2010).

Platelet activation has been extensively investigated in hematologic malignancies. Myeloproliferative disorders (MPD) are characterized by an increased risk of thrombo-hemorrhagic events, probably related to platelet dysfunction (Finazzi et al. 1996; Wehmeier et al. 1997). Platelet aggregation studies in MPD have revealed a tendency toward spontaneous platelet aggregation and a correlation between agonist-induced platelet hyperactivation and a history of thrombosis (Balduini et al. 1991). Similarly, FCM analyses showed an increased percentage of CD62P-expressing platelets, PLAs, and PMAs in the bloodstream of patients with MPD compared wth healthy controls (Jensen et al. 2000, 2001; Villmow et al. 2002).

Additionally, MPD patients with a previous history of thrombosis or microvascular disturbances had a higher percentage of platelet–granulo/monocytes and PMAs than patients with no history of these events (Jensen et al. 2001). Moreover, in vitro formation of platelet–polymorphonuclear leukocyte aggregates was enhanced in essential thrombocythemia patients without pharmacological antiaggregation but reduced in essential thrombocythemia patients treated with aspirin (Falanga et al. 2005).

Hemorrhage is one of the principal symptoms associated with acute myeloid leukaemia (AML). It may be responsible for a lethal course of the disease and is related to different factors, including thrombocytopenia and defects in platelet function (Cowan et al. 1975; Estey et al. 1982). Leinoe and colleagues demonstrated that in vitro expression of CD62P was reduced in AML patients with bleeding tendency, suggesting that FCM analysis of platelet function is a putative biological marker of hemorrhage in this disease (Leinoe et al. 2004).

AML can arise in a context of genetic predisposition, as in the case of familial platelet disorder with a predisposition to acute myelogenous leukemia (FPD/AML), an autosomal dominant platelet disorder characterized by thrombocytopenia, platelet function defects, and a lifelong risk of the development of hematologic neoplasms. The disorder is associated with germline heterozygous mutations in the transcription factor gene *RUNX1*. Analysis of defective $\alpha_{IIb}\beta_3$-dependent activation pathways has been documented as reduced binding of FITC-conjugated PAC-1 and Alexa Fluor 488-conjugated fibrinogen (Glembotsky et al. 2014).

Monitoring of Antiplatelet Therapies

FCM is a useful tool for monitoring antiplatelet therapies. Medical therapy to reduce platelet activation is the mainstay in prevention of atherothrombotic events in many vascular diseases. Several antiplatelet drug are available, the most popular being aspirin and clopidogrel (a $P2Y_{12}$ inhibitor), but other $P2Y_{12}$ and GPIIb/III antagonists have been developed, including prasugrel, ticagrelor, and tirofiban (Ford 2015; Rollini et al. 2016; Savonitto et al. 2015). Clinical trials assessing the efficacy of these drugs have been mainl

based on evaluation of platelet reactivity using FCM analysis of both surface antigens and intracellular VASP.

VASP assay, based on FCM, is a reliable index of platelet ability to be activated by ADP. The degree of VASP phosphorylation can be measured in a direct, cost-effective, and automated manner as described above (see "Intracellulars Markers of Platelet Activation"). This method has been used to detect clopidogrel resistance and to compare the efficacy of different $P2Y_{12}$ inhibitors (Aleil et al. 2005; Bednar et al. 2015).

Detection of surface marker CD62P is used to evaluate the contribution of antithrombotic therapy on platelet activation after acute coronary syndrome and after coronary intervention (Gawaz et al. 1996; Neumann et al. 1997a; Ault et al. 1999). Other activation markers can be utilized, usually in combination with P-selectin, to monitor antiplatelet therapies. Indeed, results from the PRINCIPLE-TIMI 44 study show a correlation between platelet reactivity before and after $P2Y_{12}$ blockade, as assessed by FCM measurement of CD62P and PMAs (Frelinger et al. 2011). Moreover, we have described an FCM-based method for assessing the effects of clopidogrel and tirofiban on platelet activation using 5 μL of whole blood as starting material. Peripheral blood samples were collected from patients before drug administration and after 2, 6, and 24 h of treatment with tirofiban and clopidogrel alone and in combination. Pharmacological treatment was able to induce modulation of platelet reactivity to ADP-induced activation, as assessed by PAC-1 and P-selectin (Solinas et al. 2009).

Monitoring platelet reactivity is extremely relevant, not only in CAD patients but also in the setting of ischemic stroke, in which it is well established that antiplatelet therapy reduces the frequency of secondary events (Hennekens 2002; Smith et al. 1999; Serebruany et al. 2004, 2005; Yip et al. 2004; Grau et al. 2003; Klinkhardt et al. 2003; Moshfegh et al. 2000). FCM detection of CD62P and CD63 expression shows that platelet activity is significantly more suppressed in patients on clopidogrel than in those taking aspirin in the subacute and convalescent phases of non-cardioembolic ischemic stroke. No time-dependent modulation of CD62P expression could be detected in patients on anticoagulants (warfarin) (Tsai et al. 2010; Yip et al. 2004). An innovative and promising use of FCM is documented by Serebruany and coworkers, combining aggregometry tests with FCM determination of 14 platelet surface receptors. The authors found a marked heterogeneity of platelet characteristics in patients after ischemic stroke, suggesting that bleeding complications and hemorrhagic transformation after aggressive antiplatelet regimens could be related to the decreased or normal baseline platelet characteristics in such patients (Serebruany et al. 2004).

Diagnosis of Platelet Function and/or Number Disorders

Among the clinical applications of FCM platelet analysis, diagnosis of specific platelet function disorders (PFDs) is one of the most relevant. PFDs encompasses a heterogeneous group of both inherited and acquired diseases that affect platelet function and/or number and lead to a defective primary hemostasis. Disorders include Bernard-Soulier syndrome (BSS), Glanzman thromboasthenia (GT), platelet-type von Willebrand disease, storage pool diseases, Scott syndrome, heparin-induced thrombocytopenia (HIT), immune-mediated thrombocytopenias, and rare inherited conditions associated with somatic defects, such as MYH9-related diseases and thrombocytopenias associated with skeletal defects (Carubbi et al. 2014b).

The diagnostic laboratory work-up for PFDs is challenging and involves several methodologies, including bleeding time, light transmission or impedance aggregometry (the leading assay for investigating PFDs), PFA-100, lumiaggregometry, HPLC, fluorescence microscopy, TEM, ELISA, and radioimmunoassay (Paniccia et al. 2015).

The main drawbacks of these tests are a high false-positive rate (~20 %), unreliability in cases of platelet count below 50×10^9/L, lack of standardization, and variation in result interpretation. Additionally, their use is still limited to specialized laboratories (Pai and Hayward 2009; Miller 2009).

In this scenario, FCM is a rapid, reliable, and feasible technique for the diagnosis of PFDs characterized by surface glycoprotein deficiency (e.g., GT and BSS, for which it is the method of choice), storage pool disease, and Scott syndrome (Carubbi et al. 2014b; Nurden and Nurden 2014).

In more detail, diagnosis of BSS is based on the demonstration of GPIb-IX-V deficiency by specific monoclonal antibodies targeting glycoproteins Ib, IX, and V. By this means, abnormalities in the complex structure and levels of expression can be detected, also allowing discrimination between homozygous and heterozygous states (Andrews and Berndt 2013).

In GT, FCM analysis of $\alpha_{IIb}\beta_3$ integrin expression by monoclonal antibodies recognizing GPIIb (CD41) and GPIIIa (CD61) is used as a confirmatory diagnostic tool. FCM can determine the levels of expression of $\alpha_{IIb}\beta_3$ per platelet, allowing GT patients to be subclassified as having type I, II, or III disease according to the amount of $\alpha_{IIb}\beta_3$ present per platelet (respectively <5 %, 10–20 %, or equal to 50 % of the normal amount) (Nurden et al. 2012). Moreover, in heterozygous patients, this methodology can establish $\alpha_{IIb}\beta_3$ levels in various platelet populations and, consequently, whether the clinical picture is the result of a global reduction in antigen expression by all platelets or the

coexistence of normal platelets and platelets lacking $\alpha_{IIb}\beta_3$ (Sharp et al. 1998).

Auto- and allo-antibodies or paraproteins directed against $\alpha_{IIb}\beta_3$ can mimic inherited GT and are responsible for so-called acquired GT (aGT). aGT is an extremely rare bleeding disorder whose diagnostic work-up relies on complex and time-consuming laboratory assays that demonstrate the presence of circulating proteins interfering with platelet function. The assays include aggregation tests, platelet adhesion to a collagenated surface, ELISA, and mixing studies. Giannini and coworkers describe an FCM-based method for investigating aGT by assessing PAC-1 and fibrinogen binding to patient's platelets (absent), expression of the $\alpha_{IIb}\beta_3$ complex on patient's platelets using different monoclonal antibodies against GPIIb (clones SZ22, P2, A2A9/6; decreased) or GPIIIa (clones SZ21, SAP; normal), and PAC-1 and A2A9/6 binding to control platelets in the presence of patient's serum (reduced). Overall, FCM emerged as the only test able to characterize both the functional effect and the molecular target of the patient's autoantibody on platelets (Giannini et al. 2008).

FCM has expedited the diagnostic procedure for δ-storage pool disease. FCM assay relies on the same principles as fluorescence microscopy (for decades the gold standard in the diagnosis of these disorders), detecting the fluorescence of mepacrine, a dye that selectively binds to adenine nucleotides stored in δ-granules (Gordon et al. 1995).

FCM can rapidly and effectively detect Scott syndrome platelet abnormality, characterized by defective scrambling of membrane PS, which fails to be exposed on the outer membrane leaflet after platelet activation. This defect can be feasibly identified by the annexin V binding test, which is based on annexin V binding to phospholipid exposed after activation and can reveal a lack of PS exposure (Zwaal et al. 2004).

Experimental applications of FCM in PFDs include assessment of serotonin release in δ-granule storage disease (Gobbi et al. 2003), quantification of von Willebrand binding induced by ristocetin to fresh or formalin-fixed donor platelets for differential diagnosis between platelet-type von Willebrand disease and von Willebrand disease (Giannini et al. 2007, 2010), and analysis of defective platelet activation pathways in the rare FPD/AML disorders (Glembotsky et al. 2014).

Additionally, FCM can offer a simple, easy-to-perform diagnostic pre-test for patients with a bleeding history suggestive of PFD, as proposed by Dovlatovla and colleagues. The authors describe an FCM-based platelet function test to select, among patients with excessive bleeding, those that would benefit from further, extensive platelet phenotyping. The sample is stabilized for up to 9 days using a fixing solution (PAMFix; Platelet Solutions, Nottingham, UK) and then tested for P-selectin, as a marker of α-granule secretion and a general indicator of platelet reactivity, and for CD63 to evaluates δ-granule secretion after stimulation with combinations of ADP, the thromboxane A_2 analog U46619, arachidonic acid, epinephrine, and TRAP. The assay shows good agreement with conventional lumiaggregometry and could thus be an appealing screening tool in this complex clinical scenario (Dovlatova et al. 2014).

FCM currently holds a marginal but expanding role in the routine diagnostic work-up of acquired thrombocytopenias such as immune-mediated thrombocytopenias and heparin-induced thrombocytopenia (HIT). In the case of HIT, FCM rapidly detects platelet activation induced by heparin-dependent cell-activating anti-PF4/heparin antibodies by annexin V binding (Tomer 1997), serotonin release (Gobbi et al. 2003), CD62P expression (Vitale et al. 2001), and platelet microparticle formation (Mullier et al. 2010).

Immune-mediated thrombocytopenias include a wide group of disorders typified by a reduction in platelet number as a result of production of antibodies directed against self-platelet antigens (immune thrombocytopenia; ITP) or neonatal/donor platelet antigens, most commonly human platelet antigen (HPA)-1a, as in the case of neonatal alloimmune thrombocytopenia, (NATP) and post-transfusion purpura (PTP). Innovative FCM assays have been developed to identify circulating autoantibodies and platelet-bound autoantibodies in ITP (Tomer et al. 2005; Tomer 2006) and to detect human leukocyte antigen-directed antibodies in the case of alloimmunization after repeated platelet transfusions (Carrick et al. 2011). FCM has proven to be a very sensitive method for detection of alloantibodies, as it is capable of detecting very small amounts of platelet-bound antibodies.

Interestingly, Freliger and colleagues utilized FCM-based platelet activation tests to investigate bleeding tendency in ITP patients. The following markers of activation were considered: CD62P expression, PAC-1-binding, and TRAP-stimulated platelet surface CD42b. The authors conclude that unstimulated platelet surface P-selectin and CD42b expression, together with higher levels of immature platelet fraction and platelet forward light scatter, were associated with a higher bleeding score, independently of platelet count, and could consequently be considered FCM markers of bleeding risk in ITP patients (Frelinger et al. 2015).

In the diagnostic algorithm of NATP, FCM is used to test maternal serum against paternal and maternal platelets and a small panel of platelets from normal group O donors typed for selected common HPA antigens. In the assay, washed platelets are sensitized with maternal or control serum for up to 60 min at room temperature. Platelets are then carefully washed to remove nonspecific immunoglobulins. Platelet-bound antibodies are detected with a fluorescently labeled

(usually FITC) polyclonal or monoclonal antibody specific for human immunoglobulin. Results are expressed as the ratio of the fluorescence (mean or peak) emitted by normal platelets sensitized with maternal serum to that emitted by normal platelets incubated in normal serum. To prevent nonspecific binding of the immunoglobulin probe via Fc receptors on the target platelets, the probe antibodies are enzyme-treated to remove the Fc end of the molecule. A second fluorescent label, usually phycoerythrin (PE), can be attached to an anti-human IgM probe to detect IgM anti-platelet antibodies. Tests can be run simultaneously on the same sample of washed sensitized platelets, allowing detection of anti-platelet IgG and IgM in the course of the same acquisition (McFarland 2003; Peterson et al. 2013; Curtis and McFarland 2009). The same assay also applies to PTP, as described in the section "Platelet Crossmatching".

A common potential drawback of the above-described FCM-based method of detection of serum alloantibodies is the fact that it does not differentiate between platelet-specific (i.e., platelet glycoprotein-directed) and non-platelet-specific (i.e., human luekocyte antigen- and ABO-directed) antibodies, leading to potential non-platelet-specific reactivity.

In addition to the detection of circulating anti-HPA-1a antibodies, FCM has been proposed as a rapid, simple, and reliable tool for platelet immunophenotyping in the setting of large-scale screenings to identify HPA-1a-negative subjects, potentially at risk of NATP, PTP, and refractoriness to platelet transfusion (Killie et al. 2004; Sorel et al. 2004; Tazzari et al. 1998). Of these three alloimmune disorders, NATP requires parental platelet antigen typing when a personal obstetric history suggestive of NATP is present, or in the case of maternal sister(s) with an obstetric history of laboratory-confirmed NATP or suggestive of NATP.

FCM-based platelet immophenotyping relies on the fact that polyporphisms of the HPA-1 system antigen, which account for alloimmune thrombocytopenias, result in different expression of binding sites for monoclonal antibodies of platelet membrane glycoproteins. Weiss and coworkers (Weiss et al. 1995) first characterized a monoclonal antibody, SZ21, directed against GPIIIa and able to distinguish between HPA-1a and HPA-1b genotypes because of its markedly reduced binding to platelets with the HPA-1b genotype. Starting from this finding, Schwippert-Houtermans and coworkers developed and standardized an FCM method for classifying the HPA-1 genotype, utilizing SZ21 monoclonal antibody (Schwippert-Houtermans et al. 2001). This method was subsequently implemented by coupling SZ21 with P2 antibody (targeting the $\alpha_{IIb}\beta_3$ complex), which allows discrimination of HPA-1a/1a phenotype from HPA-1a/1b thanks to absence of overlapping of P2/SZ21 mean fluorescence intensity ratios between the two phenotypes (Sorel et al. 2004).

FCM analysis using immunofluorescence labeling with specific alloantisera has been described (Forsberg et al. 1995; Tazzari et al. 1998), but is not able to distinguish HPA-1a/1b hetererozygous from HPA-1a homozygous subjects.

Today, the development of DNA-based methods allowing simultaneous genotyping of as many as 17 HPAs has made serological typing for HPA antigens obsolete (Bertrand and Conti 2015).

Blood Bank Applications

FCM is available in platelet blood banking, especially for (1) enumeration of residual white blood cells in red blood cells and platelet concentrates (PCs); (2) determination of platelet function in PCs by measuring platelet activation markers; (3) sterility testing of PCs to assess bacterial risk; and (4) platelet crossmatching.

Enumeration of Residual White Blood Cells in Red Blood Cells and Platelet Concentrates

FCM methods for counting residual white blood cells (WBC) in PCs are well established and widely used (van der Meer et al. 2012; Fischer et al. 2012). Traditionally, the methods are based on propidium iodide DNA staining for WBC detection and on fluorescent beads, at known concentration, as internal standard for leukocyte enumeration (Barclay et al. 1998; Backteman et al. 2002; Dijkstra-Tiekstra et al. 2004; Santana and Dumont 2006). Other FCM methods have also been proposed; for example, using a fluorescent anti-glycophorin A antibody that allows the simultaneous enumeration of residual WBCs and residual RBCs (Schmidt et al. 2009), and the use of specific WBC fluorescent antibody to discriminate WBCs from other nucleated cells (i.e., nucleated red blood cells) that are responsible for the overestimation of WBC content (Fischer et al. 2012).

Determination of Platelet Function in Platelet Concentrates by Platelet Activation Markers

Platelet transfusions are routinely used as life-saving procedures during surgery, myeloablative therapies, and in patients with particular bleeding disorders. The primary objective of platelet transfusion is to provide a sufficient amount of platelets with preserved hemostatic function. Unfortunately, platelet storage and pathogen reduction technologies (PRTs) cause a decrease in functionality over time, often referred to as "platelet storage lesion" (PSL)

(Thon et al. 2008). In vitro platelet function tests, mainly FCM tests, have been used to determine the platelet reactivity of PCs during storage and to compare it with that of PCs prepared with different PRTs.

Although FCM detection of platelet-derived extracellular vesicles and LAMP-1 (lysosme-associated membrane protein-1) have been proposed as new markers of PSL (Black et al. 2015; Sodergren et al. 2015; Pienimaeki-Roemer et al. 2014), the assessment of platelet functionality in PCs primarily relies on the evaluation of activation-dependent changes in platelet surface markers in a resting state and after agonist stimulation (Cardigan et al. 2005). The physiological increase in CD62p, $\alpha_{IIb}\beta_3$, and fibrinogen binding in response to platelet agonists is hampered during platelet storage (Curvers et al. 2004; Lozano et al. 1997; Rock et al. 2003; Leytin et al. 2004), whereas GpIII expression is enhanced. This increase, measured both by western blot and FCM, is a result of translation mechanisms that, in turn, may be involved in the initiation or exacerbation of PSL (Thon and Devine 2007).

Several reports now associate GPIbα shedding and increased expression of CD63 and PS to augmented platelet clearance, leading to reduced post-transfusion platelet survival (Ohto and Nollet 2011; Canault et al. 2010; Albanyan et al. 2009a, b; Metzelaar et al. 1993).

A controversial aspect of FCM application in PC storage is the evaluation of P-selectin expression for quality control. Because P-selectin expression is the most commonly applied parameter of platelet activation in PCs stored in blood banks, efforts have made to standardize its measurement (Middelburg et al. 2013; Curvers et al. 2008). Expression of P-selectin is widely used as predictor of platelet survival and function in vivo; furthermore, FCM detection of CD62p is often used to study the effects of pathogen reduction strategies on PSL (Ostrowski et al. 2010; Galan et al. 2011; Johnson et al. 2011; Castrillo et al. 2013; Ignatova et al. 2015).

However, some authors debate the use of P-selectin as a predictor of platelet survival in vivo, providing data in support of the fact it does not mediate platelet clearance. Specifically, in a nonhuman primate model, infused degranulated platelets rapidly lose surface P-selectin to the plasma pool but continue to circulate and function in vivo (Michelson et al. 1996). Moreover, platelets from wild-type and P-selectin knockdown mice had identical life spans, and, in a thrombocytopenic rabbit model, thrombin-activated human platelets that lose surface P-selectin survive and are effective in the rabbit circulation for as long as fresh human platelets (Berger et al. 1998; Krishnamurti et al. 1999).

Future studies are needed to clarify how PC studies translate to platelet function in vivo after transfusion (Cardigan et al. 2005).

Sterility Testing of PCs to Assess Bacterial Risk

One of the most frequent infectious complications in platelet transfusion therapy is related to bacterial contamination of PCs. To preserve platelet function, PCs are stored at room temperature and this facilitates bacterial contamination (Vollmer et al. 2012). Several works have demonstrated that sterility testing of PCs by FCM is a feasible approach both for buffy coat-derived PCs and PRP-derived PCs (Mohr et al. 2006a, b; Schmidt et al. 2006a, b; Lee et al. 2012). Traditionally, TO staining is used for detection of bacteria and, before testing, it is suggested that the PC sample is incubated for 20–24 h at 37 °C, which should be prolonged for up to 2 days for slow-growing bacteria (Mohr et al. 2006a). This inevitably protracts the assay results. In 2009, Dreier and coworkers introduced an FCM-based assay (BactiFlow) that fulfils the requirements for a point-of-issue testing of PCs with a time-to-result of approximately 1 h, combined with a high sensitivity of 150 colony forming units/mL (Dreier et al. 2009). This method was subsequently implemented as a routine method for the identification of contaminated PCs (Vollmer et al. 2011). Multicenter studies have since validated BactiFlow as a very convenient test for PC bacterial screening (Vollmer et al. 2012).

Platelet Crossmatching

Transfusion of crossmatch-compatible platelets is a consolidated strategy for transfusion of alloimmunized patients, who show refractoriness to platelet transfusion (Rebulla 2005; Rebulla et al. 2004).

Serum from alloimmunized patients can be crossmatched with platelets from PCs already available for transfusion or with frozen or refrigerated aliquots of platelets from potential donors. HLA-specific and HPA-specific antibodies in the patient's plasma react with platelets expressing incompatible antigens. Only platelet components that are compatible are transfused (Stroncek and Rebulla 2007). Several methods have been used to crossmatch patient samples, including ELISA, platelet immunofluorescence (PIFT), solid phase red cell adherence assay (SPRCA), and FCM. In FCM tests, donor platelets are incubated with a patient's serum or plasma. Binding of the patient's antibodies on donor platelets is detect using fluorescently labeled anti-human IgG antibodies. A platelet-specific monoclonal antibody (such as anti-CD41) should be added so that the analysis is performed exclusively on the platelet population (Sayed et al. 2011). The test must include the donor serum as negative control. Data are usually presented as the fluorescence ratio, defined as the ratio between the fluorescence intensity of gated platelets after incubation with the patient's serum and the fluorescence intensity of the negative control

(Dohlinger et al. 2005; Sayed et al. 2011). It has been demonstrated that platelet donor selection using FCM crossmatch gives a better clinical outcome for transfusion in many thrombocytopenic alloimmunized patients (Sintnicolaas and Lowenberg 1996; Sayed et al. 2011). Moreover, this method has proved useful for comparing the detection of platelet antibodies in fresh and frozen cells in attempts to standardize the storage of donated platelets (Dohlinger et al. 2005).

Other Applications

Intraplatelet Production of Reactive Oxygen Species

Platelet aggregation is associated with considerable production of reactive oxygen species (ROS), which is not counterbalanced by adequate intracellular content of antioxidants (Krotz et al. 2004). Generation of intraplatelet ROS occurs in both physiological and pathological conditions (Ghoti et al. 2007; Amer et al. 2005; Becatti et al. 2013; Mondal et al. 2015) and has been implicated in the regulation of $\alpha_{IIb}\beta_3$ activation, granule secretion, platelet shape change, and, more generally, in platelet reactivity (Begonja et al. 2005).

Detection and quantification of ROS production is relevant for measurment of platelet oxidative stress, and FCM offers a simple and rapid assay for this. The technique essentially relies on the incubation of platelets with dyes that can diffuse across the cell membrane. In basal conditions the dyes are not fluorescent, but, when oxidized by ROS, they emit a fluorescent signal that is detected by the flow cytometer. The most commonly used dyes are 2′,7-′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) and dihydrorhodamine 123 (DHR). Fluorescence is measured in the FL1 green channel. The test can be performed directly on whole blood.

Calcium Flux

Cytosolic free calcium ions (Ca^{2+}) are important second messengers and markers of platelet activation and reactivity. Therefore, quantitative measurement of intraplatelet calcium provides additional information for evaluation of platelet status. Traditionally, Ca^{2+} dynamics has been studied by cuvette-based methods, whose main drawback is the necessity of platelet isolation steps that are not only time consuming, but can also interfere with platelet activation status. One of the first FCM-based methods for the assessment of intraplatelet Ca^{2+} dynamics relied on the use of Indo-1 as fluorescent dye. However, excitation of Indo-1 requires a laser in the UV range (not standard on most flow cytometers) and the staining precludes the combined use of compounds that exhibit fluorescence when excited by UV light (i.e., oxidized lipids/lipoproteins), so this method has very limited application (Dustin 2000). Other dyes have been proposed that have the advantage of being excited by a standard blue argon laser, such as fluo-3-acetoxymethyl ester (Fluo-3) and its derivative, the brighter and more photostable Fluo-4. The fluorescence of Fluo-4 can be detected in the FL1 green channel and is enhanced by binding of Ca^{2+}. Cellular loading and accumulation of these dyes results from esterase-mediated cleavage that convert the lipophilic (pro)dyes into polar membrane-impermeable forms that thereby also acquire the ability to bind Ca^{2+}. These dyes can be coupled with anti-CD61 (Labios et al. 2006) or anti-CD41 (do Ceu Monteiro et al. 1999) antibodies that identify platelet populations. Tests can be run both on PRP and whole blood.

More recently, Assinger and coworkers described a method that, combining Fluo-4 with Fura Red, (another acetoxy-methyl ester derivative whose fluorescence is decreased by Ca^{2+} binding and is detected in the FL3 red channel) can rule out an enhanced signal resulting from the presence of aggregates (Assinger et al. 2015).

Platelet Cytoskeleton Studies

Actin polymerization in filamentous form (F-actin) is an early event in platelet activation and is associated with shape change, granule centralization, and glycoprotein redistribution. Additionally, in the basal state, F-actin content varies between individuals (Oda et al. 1992) and can be increased in pathological conditions such as type 1 diabetes (Spangenberg et al. 1989).

Variations in platelet F-actin content can be rapidly detected by FCM. This method is uses fluorescent phalloidin and phallacidin derivatives for labeling and quantifying actin filaments. These phallotoxins (isolated from *Amanita Phalloides* mushroom) are bicyclic peptides that differ by two amino acid residues and can be interchangeably used as probes for F-actin. They stain F-actin at nanomolar concentrations and have similar affinities for both large and small filaments, binding in a stoichiometric ratio of one phallotoxin molecule to one actin subunit.

The most utilized fluorescent phallotoxin probes are 7-nitrobenz-2-oxa-l,3–phallacidin (NBD–phallacidin) (Oda et al. 1992), BIODIPY–phallacidin (Semple et al. 1997) and FITC–phalloidin (LaRosa et al. 1994a).

Prior to incubation with fluorescent phallotoxin probes, platelets can be blocked with unlabeled phallotoxins to reduce nonspecific staining (Semple et al. 1997). Fixation is required.

FCM and Platelet Aggregation

Platelet aggregation can be measured by a whole blood FCM assay (Fox et al. 2004). This method is based on measuring the decrease in the number of single platelets as they form aggregates in stimulated and stirred whole blood. The platelet number can be measured in small subsamples removed from the test tube at different time points to provide kinetic information on platelet aggregation. Platelets are labeled with platelet-specific antibody and the number of red cells is used as a reference for counting individual platelets.

The method is sensitive to microaggregate formation and can provide information on platelet disaggregation, although the approach is quite elaborate. A modification of this protocol has been described, in which platelets are labeled with two platelet-specific markers (one type of antibody conjugated with two different fluorocromes), mixed together, and stimulated with various platelet agonists (phorbol myristate acetate, collagene, ristocetin). The increase in events characterized by both fluorescent labels is representative of aggregate formation.

To date, the performance of this method has been tested in both experimental (mouse models) and clinical (GT patients, full-term neonates at 24-h of life) settings in which limited blood sampling is beneficial. Indeed, FCM proved to be a very promising tool for testing platelet activation and aggregation using a small amount of whole blood (De Cuyper et al. 2013; Baker-Groberg et al. 2016).

RNA Interference

It is well established that platelets contain megakaryocyte-transcribed mRNAs that are translated into proteins in response to physiological stimuli regulating platelet function (Weyrich et al. 2004). Manipulation of platelet RNA expression is a fascinating scenario for both research and clinical purposes, such as for characterization of the specific role of platelet proteins and microRNAs, and for manipulation of platelet function and lifespan to overcome platelet storage lesion in transfusion medicine.

It was previously demonstrated that FCM provides reliable assessment of the efficiency of short interfering RNA (siRNA) in cell populations (Ho et al. 2006). More recently, Hong and coworkers demonstrated, for the first time, that human platelets could be transfected with siRNA and that transfection efficiency could be assessed by FCM. The authors transfected fluorescently labeled siRNAs in human PRP and washed platelets, testing multiple transfection conditions and identifying the optimal method for measuring the fluorescence of transfected platelets and negative controls by FCM. The authors also demonstrated knockdown of the mRNA target in transfected platelets isolated by FCM cell sorting (Hong et al. 2011).

Platelet Count During In Vitro Megakaryocytopoiesis

FCM can be utilized for monitoring platelet production during in vitro megakaryocytopoiesis from purified hematopoietic stem cells. The method allows discrimination of in vitro produced platelets from cellular debris by combining calcein-AM staining (a hydrophilic molecule that confers a green fluorescence to living cells) with anti-CD41 monoclonal antibody. The simultaneous use of calibrated beads provides the absolute platelet count (Fig. 3). This technique is useful for monitoring end-stage in vitro megakaryocytic differentiation and for testing the effect of drugs on in vitro platelet production (Gobbi et al. 2007, 2009, 2013; Nurden et al. 2010; Carubbi et al. 2014a).

Animal Models

Animal models are important tools in laboratory studies. In our setting, platelet function tests in different animal species have a wide range of applications. In particular, ex vivo pharmacological and toxicological studies have been undertaken in experimental animal models, including works of primary veterinary interest and studies in animal models of human platelet-derived disease. Flow cytometry can be applied to platelet studies in animal models, with similar considerations as those described for human platelets.

Given the fact that human and murine hemostasis are overlapping processes, mouse models are by far the most utilized. Indeed, engineered mice mimicking human acquired and inherited bleeding disorders have been developed, helping to clarify the role of specific transcription factors, receptors, and intracellular proteins or the effects of drugs (Aktas et al. 2005; McKenzie and Reilly 2004; Magallon et al. 2011; Graham et al. 2009; Pitchford et al. 2005; Pozgajova et al. 2006; Strassel et al. 2007; Kato et al. 2004; Kassassir et al. 2013; Chow et al. 2010; Musaji et al. 2004).

During FCM platelet analysis in mouse models, we have first to consider that murine platelets are smaller and more concentrated than human platelets (Nieswandt et al. 2004). An FCM method for determining the number and activation state of circulating platelets from a single mouse over an extended period of time has been described, using only 5 μL of blood collected by tail cut. Platelets are identified using a specific fluorescent antibody and a known number of fluorescent beads for counting standardization. The authors

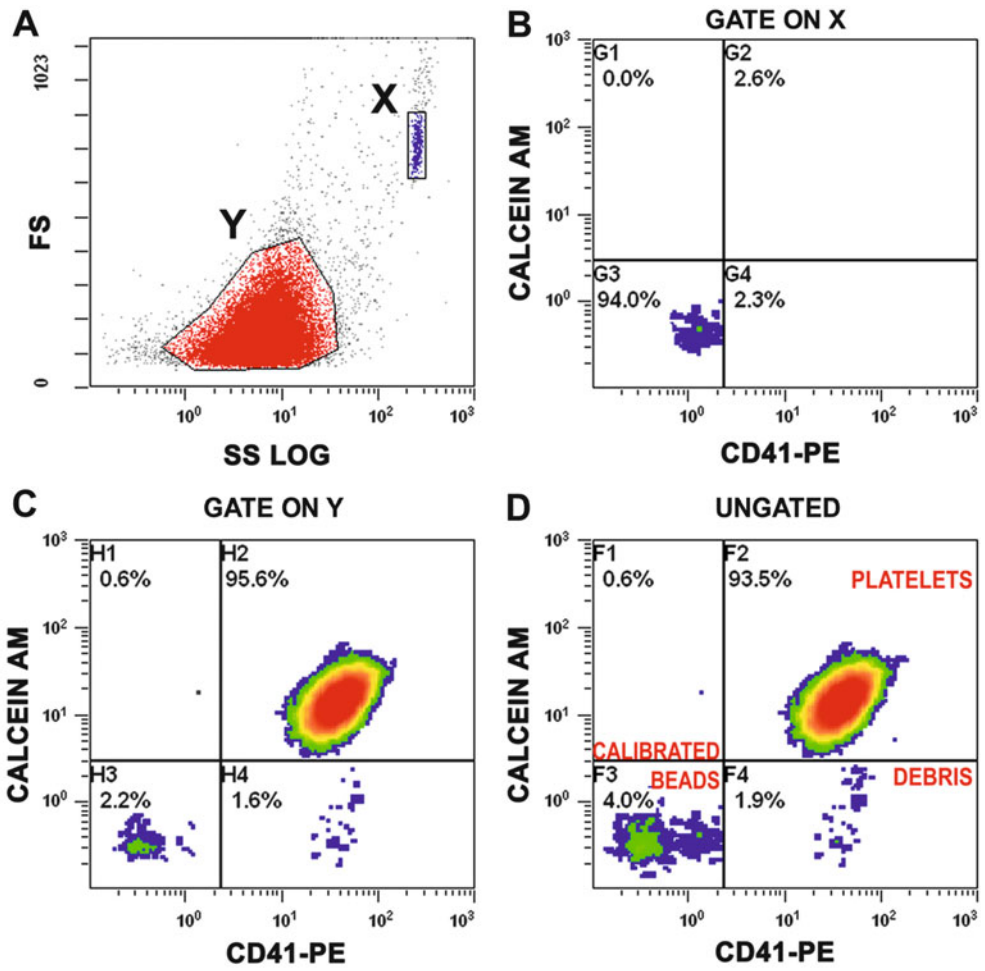

Fig. 3 Platelet quantification by flow cytometric analysis. (**A**) Distribution of platelets (*Y*) and calibrated beads (*X*) according to their forward scatter (*FS*) and side scatter (*SC*, on a logarithmic scale). (**B**) Calibrated beads appear as a well-defined and clearly distinguishable population ($CD41^-$/calcein-AM^-). (**C**) CD41 and calcein-AM staining allows discrimination between platelets ($CD41^+$/calcein-AM^+) and debris ($CD41^+$/calcein-AM^-). (**D**) Ungated plot permits calculation of platelet number according to the number of events in the *lower-left quadrant* and in the *upper-right quadrant*

demonstrated that tail vein bleeding does not activate platelets and that the method is rapid, accurate, and reproducible (Alugupalli et al. 2001).

In addition, Shipper and colleagues have validated a single-platform protocol for counting human platelets after transfusion and cord blood transplantation in the peripheral blood of NOD/SCID mice, using an anti-human CD41 antibody against human platelets and counting beads (Schipper et al. 2007).

As described for human platelets, FCM analysis of surface exposure of P-selectin and activated $\alpha_{IIb}\beta_3$ (recognized by the monoclonal JON/A antibody, equivalent to PAC-1 for murine platelets) (Bergmeier et al. 2002), as well as detection of PLAs and PMPs, are routinely utilized to assess platelet activation in vivo and ex vivo in murine models of different diseases (Pitchford et al. 2005; Ohno et al. 2014; Lamrani et al. 2014; Chen et al. 2003; Henry et al. 2009; Yokoyama et al. 2005).

It has been pointed out that the pig is a very good model for study of atherosclerosis and thrombosis (Vilahur et al. 2011). This model is being increasingly used in cardiovascular and platelet research, and a reliable method for detecting the activation of porcine platelets has been described. The authors identified a set of commercially available antibodies that bind activated platelets, also setting the optimal platelet source (whole blood or PRP) for these purposes (Krajewski et al. 2012).

FCM has also been utilized in veterinary studies investigating platelet function in various species. As an example, dogs harboring inflammatory diseases show an increase in platelet activation markers, such as PLAs and CD62p, and their expression correlates with clinical

outcome of the disease (Moritz et al. 2003, 2005; Goddard et al. 2015; Majoy et al. 2015).

More recently, the analysis of platelet surface receptors has been suggested as a useful tool in equine clinical medicine for investigation of new therapeutic strategies for the prevention or treatment of equine recurrent airway obstruction. FCM analysis has demonstrated that horses with this disease show an increase in CD41/61 and a decrease in CD62p platelet expression (Iwaszko-Simonik et al. 2015).

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Take Home Messages

- Platelet morphology and function can be comprehensively evaluated by flow cytometry
- Flow cytometry has become routinely used not only for research purposes but also in the clinic
- Relatively high costs and interlaboratory standardization are the main drawbacks that limit the application of flow cytometry

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Platelet Aggregation

Catherine P.M. Hayward and Karen A. Moffat

Abstract

Platelet aggregometry is an important technique for assessing platelet function for diagnostic and research purposes. Platelet aggregometry remains the most important, established platelet function test for evaluating platelet function for diagnostic purposes. Light transmittance platelet aggregometry and whole blood aggregometry are more popular methods for testing platelet aggregation responses than flow cytometry and platelet counting methods. In recent years, there have been considerable efforts to improve and standardize the assessment of platelet function by aggregometry. The focus has been on light transmittance platelet aggregometry as there are many more publications on this method, and more information on how the test performs for the diagnosis of bleeding disorders. Guidelines are now available on when and how to perform aggregometry (including choices of agonists, and final agonist concentrations), and how to interpret the test findings for diagnostic purposes. This chapter focuses on the principles and uses of aggregometry, with emphasis on using the test for diagnosing platelet function disorders, with proper quality control of all steps from sample procurement, processing, analysis to interpretation and reporting. Examples are provided of abnormal aggregation responses due to a variety of clinical conditions, including rare and more common platelet function disorders that impair aggregation responses. Interesting data continues to emerge on the causes of common platelet function disorders that impair aggregation responses, a number of which reflect mutations in genes encoding transcription factors that are important for generating normal, functional platelets.

Platelet aggregometry is an important technique for assessing platelet function for diagnostic and research purposes. The first descriptions of platelet aggregometry date back to more than 50 years ago, when two, independent reports by Born (1962) and O'Brien (1962) were published that described the assessment of platelet aggregation responses by light transmittance aggregometry (LTA). Later, methods were developed to test whole blood aggregation responses using changes in electrical impedance as the endpoint (Cardinal and Flower 1980; Sweeney et al. 1989a) and to test dense granule adenosine triphosphate (ATP) release simultaneously with aggregation (Feinman et al. 1977; Ingerman-Wojenski et al. 1983; Ingerman-Wojenski and Silver 1984). More than half a century later, LTA remains the most important, established platelet function test for evaluating platelet function for diagnostic purposes (Gresele et al. 2014; Cattaneo 2009). While laboratories

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continue to test LTA by the methods of Born and O'Brien, there have been considerable efforts to improve the assessment of platelet function by aggregometry, as practice surveys have highlighted the need for standardization of aggregometry tests in both research and diagnostic laboratories (Moffat et al. 2005; Jennings et al. 2008; Duncan et al. 2009; Cattaneo et al. 2009; Gresele et al. 2014). Guidelines are now available on how to perform aggregometry, and how to interpret LTA findings for diagnostic purposes (Christie et al. 2008; Hayward et al. 2010; Harrison et al. 2011; Cattaneo et al. 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015). This chapter focuses on the principles and uses of aggregometry, with emphasis on using the test for diagnosing platelet function disorders. This chapter pays particular attention to LTA as it is the best characterized and most popular aggregometry method for assessing platelet function in clinical and research laboratories (Moffat et al. 2005; Cattaneo et al. 2009; Gresele et al. 2014).

Overview and General Principles of Aggregometry

Platelet aggregometry assesses platelet–platelet interactions that are induced by adding platelet stimulating agonists to a sample. The sample tested can be whole blood (diluted or undiluted) for whole blood aggregometry (WBA) (Feinman et al. 1977; Cardinal and Flower 1980; Ingerman-Wojenski et al. 1983; Ingerman-Wojenski and Silver 1984; Swart et al. 1984; Sweeney et al. 1989a; McGlasson and Fritsma 2009), flow cytometry (Gatt et al. 2014; Rubak et al. 2015), and platelet counting methods (Lordkipanidze et al. 2009; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015). For LTA, the sample testing is usually platelet rich plasma but washed platelet suspensions can also be tested (typically only for research purposes) (Born 1962; O'Brien 1962).

The extent of platelet aggregation in response to an agonist can be quantified by measuring:

1. Turbidimetric changes for LTA (Born 1962; O'Brien 1962),
2. Changes in electrical impendence changes for WBA (Cardinal and Flower 1980; Ingerman-Wojenski et al. 1983; Ingerman-Wojenski and Silver 1984; Swart et al. 1984),
3. The loss of single platelets (due to aggregate formation) by platelet counting methods (Sweeney et al. 1989a; Lordkipanidze et al. 2009) and
4. The formation of larger aggregates, and loss of single platelets, when aggregation is quantified by flow cytometry methods (Gatt et al. 2014; Rubak et al. 2015).

LTA and WBA are the more commonly used methods for quantifying aggregation responses (Moffat et al. 2005; Cattaneo et al. 2009; Gresele et al. 2014). Because there is more information published on LTA than WBA, particularly for diagnosing platelet function disorders, the majority of laboratories use LTA for diagnosing platelet function disorders (Moffat et al. 2005; Cattaneo et al. 2009; Gresele et al. 2014).

For LTA and WBA aggregation tests, samples are stirred at low shear force (Christie et al. 2008; Cattaneo et al. 2013). Under these conditions, aggregation tests assess platelet–platelet interactions mediated by the binding of the γ chain of fibrinogen to $\alpha_{IIb}\beta_3$ receptors on adjacent platelets (Ni and Freedman 2003). For LTA, cloudiness of the sample from lipemia can interfere with quantifying the aggregation responses, as can large numbers of contaminating leukocytes, red cells, or red cell fragments in a sample (Christie et al. 2008).

More details on LTA and WBA are provided in the sections that discuss these methods for evaluating platelet function.

Preparation of Samples for Aggregometry

Process control is important for obtaining proper samples for aggregometry and controlling the analytical and post-analytical steps of platelet function testing (Hayward and Eikelboom 2007; Favaloro 2009; Hayward et al. 2012). The box below summarizes important quality assurance considerations for preparing samples to test by platelet aggregometry. Although other anticoagulants have been explored (Hellstern et al. 2007; Haubelt et al. 2008; Tauer et al. 2010; Loreth and Klose 2010; Kaiser et al. 2011; Gresele et al. 2014), most blood samples for aggregometry are collected into buffered sodium citrate anticoagulant as aggregation responses are influenced by pH and calcium chelation (Lages and Weiss 1981; Hellstern et al. 2007; Haubelt et al. 2008; Tauer et al. 2010). The most commonly used anticoagulants are 109 and 129 mM buffered sodium citrate, which increase aggregation responses to some weak agonists that are useful for diagnostic aggregometry, such as epinephrine (adrenaline) and adenosine diphosphate (ADP) (Mustard et al. 1975; Lages and Weiss 1981).

Quality Assurance Considerations for the Preanalytical Components of Aggregation Testing
The recommendations summarized are compiled from recent guidelines (Christie et al. 2008; Hayward et al. 2010; Harrison et al. 2011; Cattaneo et al. 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015).

Subject preparation

Blood samples should be collected from subjects after a short rest period. Subjects should refrain from smoking for at least 30 min, and abstain from caffeine for at least 2 h.

Medications taken during the previous week should be reviewed for ingestion of drugs that reversibly inhibit platelet function. Reversible non-steroidal anti-inflammatory drugs need to be stopped at least 3 days before samples are taken whereas aspirin and thienopyridines need to be stopped at least 10 days in advance. When drugs that inhibit platelet function cannot be stopped, their effects on platelet function need to be considered when interpreting the findings.

There is diurnal variation in aggregation responses so most laboratories schedule the testing for the morning and early afternoon.

Blood collection

Aggregometry samples should be collected with **minimal or no venostasis, using a 21 gauge or larger needle. The first 3–4 ml of blood can be discarded or used for other coagulation tests. The discard volume should be minimized or omitted for small children.**

The blood should be collected into plastic (polypropylene) **or siliconized glass tubes containing 109 mM (3.2 %) or 129 mM (3.8 %) buffered sodium citrate anticoagulant**, taking care to properly fill the collection tubes. Under-filled tubes may only be used to exclude severe platelet function disorders (i.e., **Glanzmann thrombasthenia or Bernard Soulier syndrome).**

Preparation of platelet rich and platelet poor plasma for LTA

Blood samples should "rest" at room temperature for 15 min before centrifugation.

Platelet rich plasma should be prepared by centrifuging samples at 200 g for 10 min, at ambient temperature, without using the centrifuge brake.

When the subject has thrombocytopenia and giant platelets, platelet rich plasma should be harvested without centrifugation, by allowing the sample to sediment.

Platelet poor plasma should be prepared by centrifuging whole blood, or the leftovers from the tube used to prepare platelet rich plasma, at 1500 g for 15 min, at ambient temperature.

Visual check for sample quality

Grossly hemolyzed samples should be discarded.

If the sample is lipemic, the final report should state this.

Samples of platelet rich plasma should appear uniform, pearly, and swirl when gently pipetted up and down.

Assessment of the sample

Platelet count of the aggregometry samples **should be quantified and compared to whole blood platelet counts. Normally, platelet counts are higher in platelet rich plasma compared to whole blood (typically double). The sample should have minimal leukocyte and red blood cell contamination.**

Aggregation responses can be impaired when the sample platelet count is $<150 \times 10^9$/l.

Low platelet count samples are useful to exclude some platelet function disorders associated with thrombocytopenia such as variant Glanzmann thrombasthenia, Bernard Soulier syndrome, type 2B and platelet type von Willebrand disease.

Samples can be left without an adjustment to a standardized platelet count, or adjusted to a standardized value, based on the procedure validated by the laboratory (recent guidelines suggest not to adjust, **although one study found adjusted samples superior for diagnosing platelet disorders** (Castilloux et al. 2011)).

Aggregation is typically evaluated at 37 °C during the test to mimic normal physiology (Born 1962; O'Brien 1962; Christie et al. 2008; Cattaneo 2009; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015).

Light Transmittance Platelet Aggregometry

LTA is a turbidimetric-based method that quantifies alterations in the optical density of a platelet suspension (placed in a clear receptor) that follow the addition of a platelet-activating agonists to a stirred platelet suspension, as first described by Born and O'Brien (Born 1962; O'Brien 1962). There are a number of commercial instruments that have been designed to measure LTA, and these allow for continuous monitoring of changes to the turbidity of a platelet rich plasma sample after adding an agonist. The test is

done after setting the optical limits for the sample that correspond to "no aggregation" (i.e., same turbidity as the starting sample) and full (100 %) aggregation (i.e., turbidity reduced to that of platelet poor plasma) (Cattaneo 2009). Aggregation is typically monitored for a brief period before adding the agonist to ensure that the baseline is stable (Cattaneo et al. 2013). Shortly after adding the agonist, there is often a slight reduction in the sample turbidity (from the added volume of the agonist) before platelet shape change (Sanderson et al. 1996) (which increases the turbidity), which is then followed by a reduction in turbidity due to aggregate formation (e.g., Fig. 1). Responses are usually monitored for 5 min although longer monitoring is required to reach stable final aggregation with some agonists (e.g., 10 min monitoring for epinephrine, Fig. 1) (Cattaneo et al. 2013). The monitoring should be sufficient to achieve

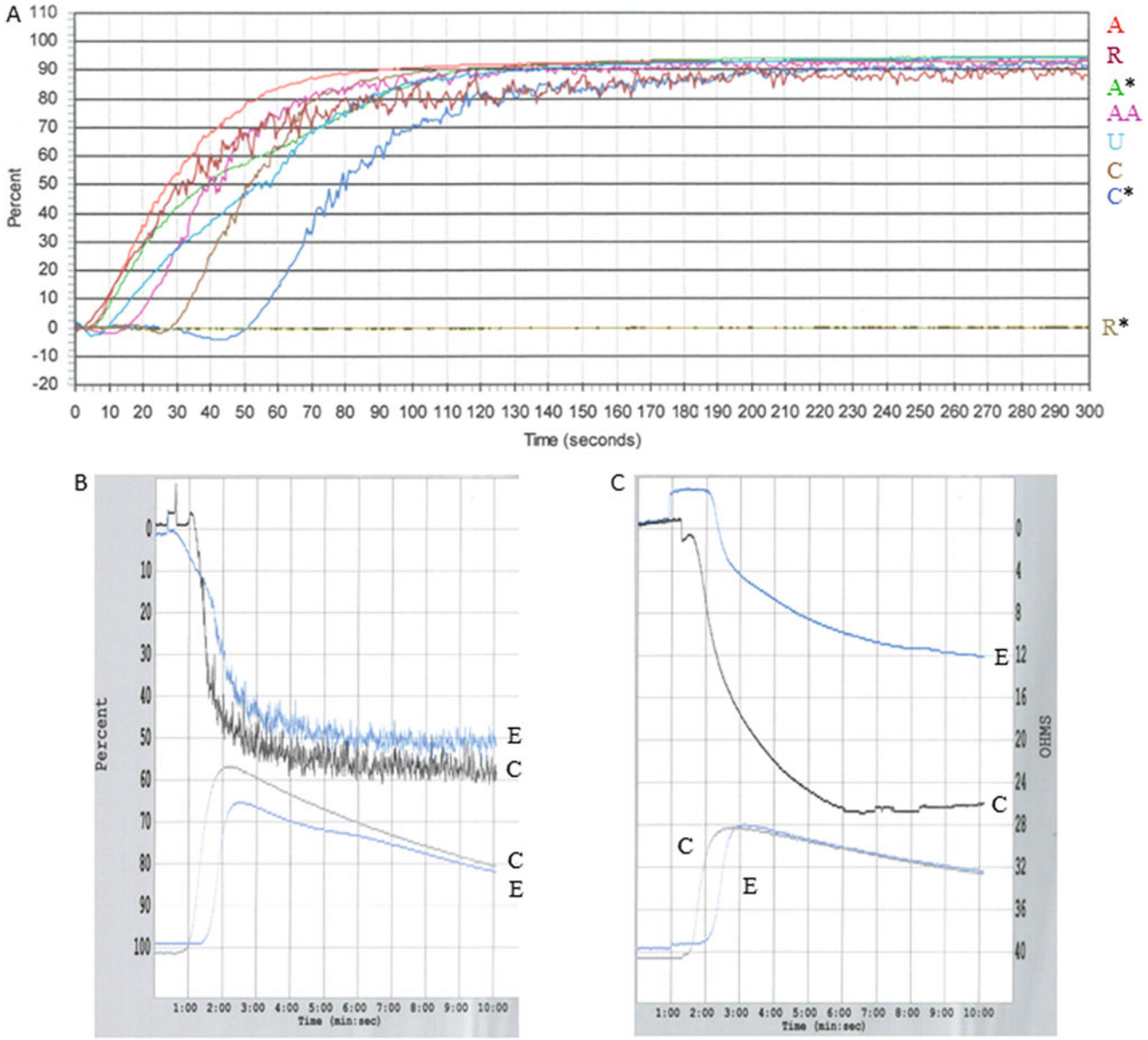

Fig. 1 Comparison of normal aggregation responses detected by light transmission and whole blood aggregometry. In the upper panel, there is no aggregation with 0.5 mg/l ristocetin (R*) and extensive aggregation with (agonist abbreviation, reference interval cutoffs): 2.5 (A*, >24 %) and 5.0 (A, >43 %) μM ADP; 1.25 mg/ml ristocetin (R, >75 %);1.6 mM arachidonic acid (AA, >77 %); 1 mM thromboxane analogue U46619 (U, >70 %); 1.25 (C*, >51 %) and 5 mg/ml collagen (C, >85 %). In the lower panels, aggregation (LTA as %; WBA in Ohms) was measured simultaneously with dense granule ATP release (nM). Shape change (increase in turbidity, before aggregation) with Horm collagen (C) is evident by LTA but not WBA. While the primary aggregation response to 6 μM epinephrine (E) occurs before dense granule release, and the start of secondary aggregation by LTA, in the WBA tracing, secretion appears to occur simultaneously with the aggregation response to epinephrine

maximal and stable aggregation responses. The maximal aggregation (maximal change in turbidity relative to the baseline) is the most important endpoint to quantify (Hayward et al. 2009). Other measures that can be reported include the final aggregation (taken at the end of the test) (Cattaneo et al. 2013) which will be lower than the maximal aggregation if there is partial or complete deaggregation (e.g., Fig. 2). The extent of deaggregation can also be quantified by subtracting the percentage final aggregation from the percentage maximal aggregation. Many instruments allow collection of additional numerical data, such as the slope or lag of the aggregation response. Numerical values should be reported along with a reference interval to aid in the interpretation of the findings.

Aggregation curves require visual inspection for proper interpretation. The baseline should be stable before adding the agonist, and tracings should be free of artifacts (e.g., abrupt changes in turbidity from a bubble in the sample, electrical interference or strong vibrations) (Cattaneo et al. 2013).

Tracings will typically show one or two phases of aggregation (Fig. 1). Biphasic LTA responses are typical of some weak agonists, such as epinephrine (Fig. 1), when buffered sodium citrate is used as the anticoagulant (Charo et al. 1977; Lages and Weiss 1980). The second wave of aggregation with epinephrine represents more rapid and extensive aggregation that occurs at the time of dense granule release and thromboxane A_2 generation (Fig. 1) (Charo et al. 1977; Lages and Weiss 1980). Biphasic responses to ADP are normal, but when this agonist is tested at recommended concentrations (e.g., 2–5 μM), the biphasic response may not be evident unless the ADP concentration is titrated downward (in Fig. 2, a more rapid, second wave of aggregation with 5 μM ADP starts at approximately 130 s).

There are differences in LTA responses to epinephrine and ADP for native (undiluted) compared to platelet count adjusted samples whereas the responses to other agonists are very similar (Mani et al. 2005; Cattaneo et al. 2007; Castilloux et al. 2011). Accordingly, LTA findings need to be reported with a reference range that was determined for the specific sample type evaluated. While biphasic aggregation with epinephrine is normal for both sample types, absent secondary aggregation with epinephrine is only abnormal for native samples (Castilloux et al. 2011). Accordingly, laboratories need to know the range of aggregation response patterns expected for healthy control samples, for the specific methods that they use, when they are interpreting the findings.

Aggregation tracings should be routinely inspected for evidence of any de- or disaggregation, which can be quantified (percentage difference between maximal and final aggregation) (Cattaneo et al. 2013). Extensive deaggregation is usually only seen when there is significantly reduced maximal aggregation (Fig. 2). Some deaggregation can be normal with low concentrations of ADP (e.g., 1–2.5 μM final), but it is usually not evident with other agonists, if they are tested at recommended concentrations (e.g., concentrations shown in Table 1). When aggregation responses are delayed, the maximal aggregation achieved is usually reduced.

When a biphasic response is observed with ristocetin, the curve should be inspected carefully as this often reflects a delay in the initial agglutination response (e.g., as seen with significant VWF deficiency, Fig. 3) (Dowling et al. 1975). If there is an impairment in the aggregation response that follows ristocetin-induced agglutination (e.g., as seen in some platelet function disorders, including Glanzmann thrombasthenia, Fig. 4), the maximal aggregation achieved is reduced.

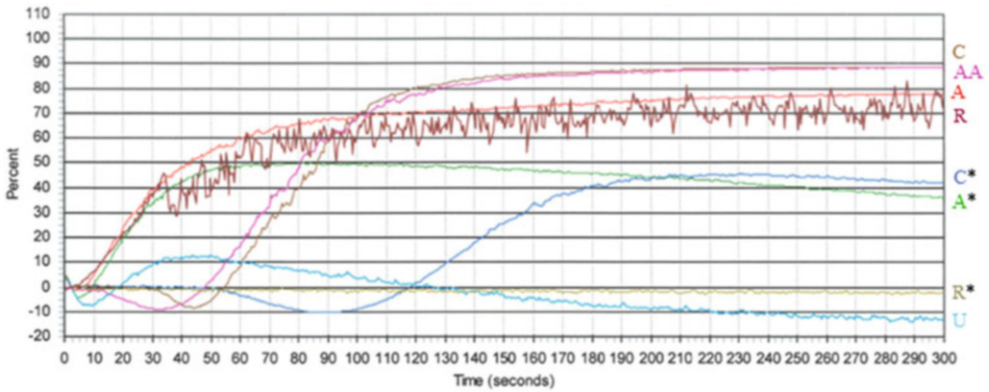

Fig. 2 Example of abnormal aggregation, and deaggregation accompanying reduced maximal aggregation. The subject has a platelet function disorder that is associated with reduced maximal aggregation (abbreviation for agonist, reference interval), and full deaggregation with 1 μM thromboxane analogue U46619 (U, >70 %), in addition to reduced aggregation with 1.25 mg/ml collagen (C*, >51 %) and 1.25 mg/ml ristocetin (R, >75 %). However, there is normal aggregation with 2.5 (A* >24 %) and 5.0 (A, >43 %) μM ADP; 5 mg/ml collagen (C, >85 %); 1.6 mM arachidonic acid (AA, >77 %); and 0.5 (R*, <7 %) and 1.25 mg/ml ristocetin (R, >75 %)

Table 1 Summary of agonists, receptors, and pathways, and recent recommendations on agonist concentrations for testing platelet responses by light transmission aggregometry

Agonist	Receptor(s) and pathway	Concentrations		Additional comments
		Commonly used	ISTH-recommended	
Adenosine diphosphate	Activation through $P2Y_1$ and $P2Y_{12}$, followed by aggregation mediated by $\alpha_{IIb}\beta_3$	2–10 μM	2 μM	Test higher concentration if the response is impaired
Epinephrine	Activation through the α_2 adrenergic receptor (A2AR), followed by aggregation mediated by $\alpha_{IIb}\beta_3$	2.5–20 μM	5 μM	Test higher concentration if the response is impaired
Collagen	Adhesion to α2β1 (adhesion) and activation via GPVI, followed by aggregation mediated by $\alpha_{IIb}\beta_3$	1–5 μg/ml type 1 collagen	A concentration that induces sufficient aggregation of normal platelets (e.g., 2 μg/ml Horm or type 1 collagen)	Test higher concentration if the response is impaired
Arachidonic acid	Requires conversion by cyclooxygenase 1 (COX1) and thromboxane A_2 synthase before activating platelets through the thromboxane A_2 receptor (TP), leading to aggregation mediated by $\alpha_{IIb}\beta_3$	1–1.6 mM	1 mM	Test higher concentration if response is impaired
Thromboxane A_2 mimetic U46619	Thromboxane A_2 receptor (TP) and aggregation mediated by $\alpha_{IIb}\beta_3$	1–2 μM	1 μM	Test higher concentration if response is impaired
Ristocetin	Agglutination of platelets through the binding of von Willebrand factor to GPIbIXV, followed by aggregation mediated by $\alpha_{IIb}\beta_3$	High: 1.2–1.5 mg/ml Low: 0.5–0.6 mg/ml	High: 1.2 mg/ml Low: 0.5–0.7 mg/ml	Test both concentrations or test the low concentration after verifying there is a response to the higher concentration
Thrombin receptor activating peptide (PAR1-AP; TRAP-6)	Protease-activated receptor 1 (PAR1)	5–20 μM	10 μM	Test higher concentration if response is impaired
γ-thrombin (free of α-thrombin)	PAR1 and PAR4	0.5–1.0 U/ml	–	γ-thrombin does not bind to fibrinogen

The final agonist concentrations that are commonly used (25–75 % percentiles for concentrations that laboratories reported using in Cattaneo et al. 2009) and the concentration that are recommended in the 2013 ISTH guidelines by Cattaneo et al. (2013) are summarized

LTA responses to collagen are more complex than with other agonists as the initial phase reflects platelets adhering to, and being activated by collagen fibrils (Jarvis et al. 2002). This results in a more pronounced "shape change" before full aggregation occurs (Fig. 1). At lower collagen concentrations, the shape change and aggregation responses occur more slowly (e.g., Fig. 2).

Whole Blood Aggregometry

WBA is typically assessed using samples of whole blood that are first diluted 50:50 with saline, a step that can be omitted when the sample has a low platelet count (Mengistu et al. 2009). Aggregometry is measured in Ohms of impedance, reflecting the electrical changes that occur when aggregating platelet attach to the electrode, which follows the attachment of a platelet monolayer (Cardinal and Flower 1980; Ingerman-Wojenski et al. 1983; Ingerman-Wojenski and Silver 1984; Swart et al. 1984; Lehmann et al. 1985; Sweeney et al. 1989b; Seyfert et al. 2007; McGlasson and Fritsma 2009). The method does not detect platelet shape change. The timing of release, relative to aggregation, can be more rapid in WBA (Ingerman-Wojenski et al. 1983). Biphasic aggregation responses are often more difficult to detect (Ingerman-Wojenski et al. 1983). As all blood cells are present, the function of all platelets, including those that are very large, are evaluated (Ingerman-Wojenski and Silver 1984). However, the measured WBA with collagen also reflects the attachment of leukocytes, in addition to platelets (Lehmann et al. 1985). On the other hand, responses to ADP (and some other agonists) reflect mainly platelet attachment to the electrode (Lehmann et al. 1985). Despite the differences in the way LTA and WBA are measured, the aggregation curves generally look quite similar (Fig. 1). WBA has the benefit of requiring less sample processing

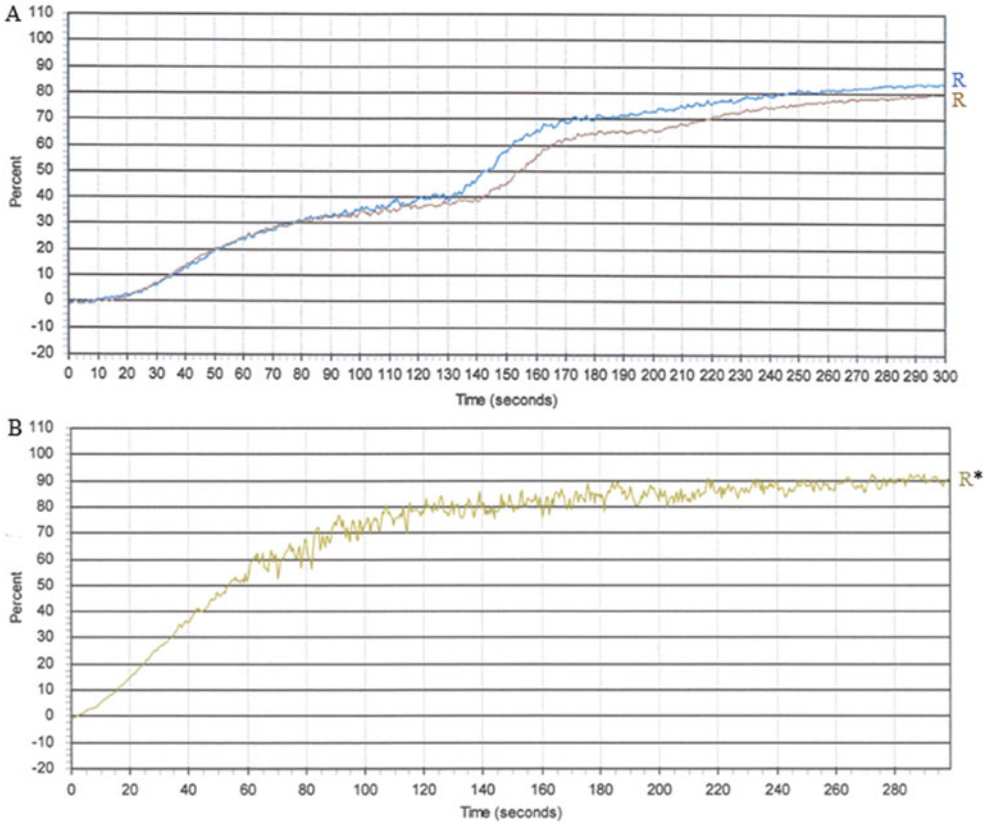

Fig. 3 Abnormal aggregation responses to ristocetin. Panel **A** shows LTA for a subject with type 2M von Willebrand disease who had a pronounced diphasic response to 1.25 mg/ml ristocetin (R, >75 %), due to the impaired agglutination. Panel **B** shows strikingly increased maximal aggregation with 0.5 mg/ml ristocetin (R*, <7 %) due to type 2B von Willebrand disease

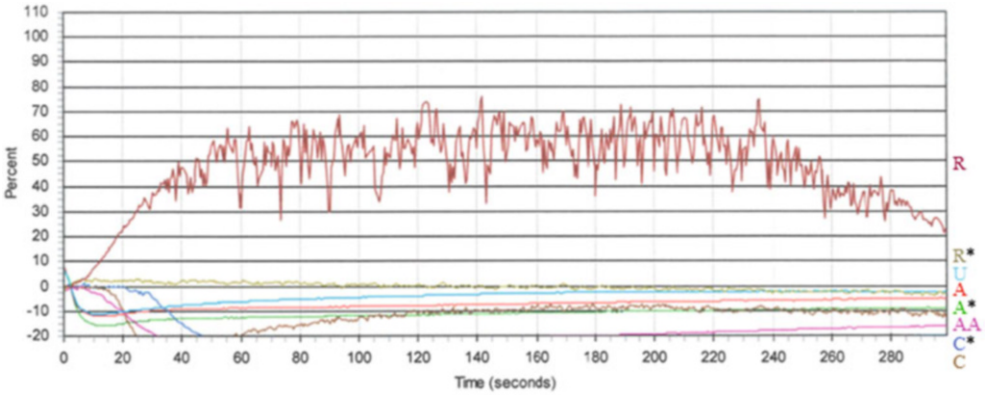

Fig. 4 Aggregation abnormalities due to Glanzmann thrombasthenia. Agglutination is present with 1.25 mg/ml (R) but not with 0.5 mg/ml ristocetin (R*). There is no visible aggregation response to: 1 μM thromboxane analogue U46619 (U); 2.5 (A*) or 5.0 (A) μM ADP; 1.25 (C*) or 5 mg/ml collagen (C); 1.6 mM arachidonic acid (AA). The response to 1.25 mg/ml ristocetin (R, >75 %) is reduced, with some deaggregation, as there is a failure to stabilize the agglutinated platelets due to absent aggregation

prior to testing and it is not affected by lipemia (Cardinal and Flower 1980; Ingerman-Wojenski and Silver 1984; Sweeney et al. 1989a, b, c; McGlasson and Fritsma 2009). Some agonists (e.g., epinephrine) are omitted when testing WBA as absent responses can be normal (Joseph et al. 1987). While LTA and WBA have been evaluated for the diagnosis of bleeding disorders (Weiss 1967, 2004; Ingerman-Wojenski and Silver 1984; Lages and Weiss 1988; Cattaneo et al. 1994; Israels et al. 2003; Hayward et al. 2009; Quiroga et al. 2009; Mezzano et al. 2009; Frontroth et al. 2010; Castilloux et al. 2011), non-inferiority or superiority of one method over the alternative has not been established.

Establishing and Verifying Aggregation Test Reference Intervals

The aggregation responses of platelets to many agonists is not normally distributed (Hayward et al. 2008) and this needs to be considered when developing reference intervals

for the test. The use of nonparametric statistical approaches is recommended as this allows for the estimation of the 95 % confidence intervals, for all types (including non-Gaussian) of data distributions (Hayward et al. 2008; Rubak et al. 2012). For LTA, maximal aggregation does not show a strong association to the sample platelet count for samples collected from donors with normal platelet counts (Castilloux et al. 2011), whereas WBA is strongly influenced by the sample platelet count (Rubak et al. 2012). For low platelet count samples, regression has been used to estimate the 95 % confidence intervals for maximal aggregation by LTA, according to the sample platelet count, for aggregometry samples containing less than 250×10^9 platelets/l (Hayward et al. 2008).

Quality Control of Aggregation Tests

Because LTA and WBA must be performed on fresh samples, ideally within 4 h of collection, the quality control of aggregation tests is often limited to testing a healthy control sample in parallel with a patient's sample, and validating new reagent lots by parallel testing with the current lot (Hayward and Eikelboom 2007; Favaloro 2009; Hayward et al. 2012; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, ISTH, 2015). Although most abnormalities are confirmed by repeat determinations (Hayward et al. 2009; Quiroga et al. 2009), it is a reasonable practice to do repeat determinations whenever the amount of aggregation falls outside the reference interval. External quality assurance exercises have been developed to evaluate aggregation test interpretations (Hayward et al. 2012). The other approach that has been tried for aggregometry monitoring is to test a sample spiked with unknown additives to induce a particular type of platelet function abnormality (Favaloro 2009).

Agonists Used to Assess Aggregation Responses

Table 1 summarizes the most commonly used agonists for platelet aggregometry (i.e., ADP, epinephrine, collagen, arachidonic acid, and ristocetin) and the concentrations of these agonists, and of the thromboxane A_2 mimetic U46619 and thrombin receptor acting peptide (PAR1-AP), that are typically used for LTA. Some agonists are used less frequently, including: the thromboxane A_2 mimetic U46619, α- or γ-thrombin, PAR1-AP, PAR 4-AP, convulxin, CRP, calcium ionophore A23187 and PMA (Cattaneo et al. 2009). Such agonists can be considered for secondary investigations of platelet function, as recommended in recent guidelines published by the ISTH (Cattaneo et al. 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015). ISTH has made specific recommendations on the final concentrations of many agonists for LTA (in the aggregation test) in an effort to reduce variations in laboratory practice (see Table 1). It should be noted that the recommended final agonist concentrations, after addition to a sample, differ for LTA and WBA and some agonists (e.g., epinephrine) are recommended for LTA but not WBA (Christie et al. 2008; Hayward et al. 2010; Harrison et al. 2011; Cattaneo et al. 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015). Evidence suggests that a limited number of agonists detect most common platelet function disorders (Hayward et al. 2009). Experts have recommended using additional agonists for secondary investigations (Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015), although the benefit of testing some of these additional agonists for the detection and diagnosis of common and rare platelet function disorders is uncertain.

The following recommended agonists bind to unique seven transmembrane domain receptors on platelets, and trigger platelet activation and aggregation: ADP, epinephrine, thromboxane analogue U46619, thrombin, and the thrombin receptor activated peptides selective for the protease-activated receptor (PAR) PAR1 or PAR 4 (Gresele and Subcommittee on Platelet Physiology of the International Society on T, Hemostasis 2015; Nurden and Nurden 2014). Collagen, convulxin, and collagen related peptide (CRP) activate platelets through glycoprotein VI and assess aggregation responses induced by glycoprotein VI signaling pathways (Jarvis et al. 2002). Horm or type 1 collagen binds to glycoprotein VI and to the platelet integrin α2β1, which augments aggregation with collagen (Jarvis et al. 2002).

Some agonists that have been recommended for evaluating platelet function lead to platelet activation and aggregation by other mechanisms. For example, arachidonic acid requires multistep, enzymatic conversion by platelet cyclooxygenase 1 (COX1) and thromboxane A_2 synthase, to activate platelets via the thromboxane A_2 receptor which is followed by aggregation (Kinlough-Rathbone et al. 1977; Khan et al. 2015). Calcium ionophore A23187 activates platelets through triggering the release of calcium from intracellular stores, which leads to aggregation (Cattaneo and Lecchi 2001). 4 beta-Phorbol-12-myristate-13-acetate (PMA) activates platelets through effects on protein kinase C, which triggers signaling that leads to aggregation (Yamaguchi et al. 1987; Jerushalmy et al. 1988). Ristocetin is an antibiotic that agglutinates but does not directly activate platelets: it triggers the binding of von Willebrand

factor to glycoprotein IbIXV, which leads to platelet agglutination, which is normally followed by platelet secretion and aggregation due to the close platelet–platelet contact induced by the agglutination (Dowling et al. 1975; Kao et al. 1979; Cattaneo et al. 1989; Sweeney et al. 1989c; Weiss 2004).

Weak and strong agonists have different effects on granule secretion and aggregation (Lages and Weiss 1988; Cattaneo 2009). Strong agonists induce more rapid aggregation than weak agonists and the maximal aggregation achieved is often more variable with weak than with strong agonists (Hayward et al. 2008, 2009; Cattaneo 2009). Weak agonists, such as epinephrine, stimulate aggregation but do not induce dense granule secretion until thromboxane A_2 generation and granule secretion occur (Fig. 1) (Lages and Weiss 1980, 1981, 1988; Cattaneo 2009). With epinephrine, this is manifested by a primary wave (initial slower aggregation) that is followed by more rapid, further aggregation (Cattaneo 2009). With other weak agonists (e.g., ADP), the primary and secondary aggregation waves often appear fused unless the agonist concentration is titrated downward until both waves are evident. Weak agonists can act synergistically, inducing more aggregation when added together than predicted by the sum of aggregation responses to the individual agonists (e.g., synergistic responses to epinephrine and ADP) (Kinlough-Rathbone et al. 1977; Vanags et al. 1992). Synergistic responses are not usually evaluated when aggregation tests are performed for diagnostic reasons. Strong agonists that induce rapid aggregation and simultaneous dense granule secretion can behave like weak agonists, if tested at lower concentrations (Cattaneo 2009). Spontaneous platelet aggregation (i.e., aggregation without an added agonist) is rarely evaluated.

Data from knockout mouse studies indicate that the extent and stability of platelet aggregates are influenced not only by fibrinogen but also by other $\alpha_{IIb}\beta_3$ ligands, such as vitronectin (Hou et al. 2015). Whether vitronectin and other $\alpha_{IIb}\beta_3$ ligands influence human platelet aggregation responses is less certain as there are no human disorder equivalents to these knockout mice.

Adhesive Receptors That Influence Platelet Aggregation

The receptors that support platelet aggregation induced by commonly used agonists are summarized in Table 1. LTA and WBA evaluate platelet–platelet interactions at low shear forces, induced by stirring of the cell suspension. The signaling pathways induced by most agonists lead to activation of the platelet integrin $\alpha_{IIb}\beta_3$, which allows fibrinogen (a dimeric protein) to bind $\alpha_{IIb}\beta_3$ receptors and leads to the formation of aggregates, as fibrinogen forms bridges between $\alpha_{IIb}\beta_3$ receptors on adjacent platelets (Holmback et al. 1996). While $\alpha_{IIb}\beta_3$ is not required for platelet agglutination mediated by ristocetin, the agglutination leads to the activation of $\alpha_{IIb}\beta_3$ (Navdaev et al. 2014). Accordingly, defective aggregation with ristocetin can reflect defects in VWF (Fig. 3), loss or dysfunction of $\alpha_{IIb}\beta_3$ (Fig. 4) or GPIbIXV (Fig. 5), or impairments in platelet activation and signaling that follow ristocetin-induced agglutination and affect $\alpha_{IIb}\beta_3$ activation (e.g., Fig. 7). Gain-of-function defects in platelet agglutination mediated by VWF binding to GPIbIXV occur in type 2B or platelet type von Willebrand disease, which selectively increases aggregation responses to low concentrations of ristocetin (Fig. 3b) (Dowling et al. 1975; Weiss 2004; Othman and Emsley 2014).

Examples of Aggregation Abnormalities Due to Inherited and Acquired Platelet Function Disorders

Defects in aggregation responses can reflect defects in receptors for agonists, adhesive receptors or their ligands, signaling pathways or complex defects. The causes of common inherited platelet disorders, which are often inherited as a dominant trait, are emerging to be caused by mutations in genes encoding transcription factors with key roles in megakaryopoiesis and the production of normal, functional platelets (Bunimov et al. 2013; Hayward 2011; Songdej and Rao 2015). Furthermore, defects that are limited to specific pathway(s) are important but less common causes of platelet function disorders (Bunimov et al. 2013; Freson et al. 2014; Nurden and Nurden 2014).

Figure 4 shows typical aggregation findings for Glanzmann thrombasthenia, which are less severe in variant Glanzmann thrombasthenia, due to activating mutations in either αIIb or β3 (Awidi et al. 2009; Nurden and Nurden 2011; Nurden et al. 2011). When $\alpha_{IIb}\beta_3$ is absent or severely impaired, agglutination is present with ristocetin whereas aggregation responses are absent with all other agonists. Similar abnormalities can be seen in subjects with strong inhibitory antibodies against $\alpha_{IIb}\beta_3$, or that have received treatment with a potent $\alpha_{IIb}\beta_3$ inhibitor.

Figure 5 shows aggregation abnormalities that are typical of Bernard Soulier syndrome (Nurden and Nurden 2011; Noris et al. 2012). In all cases, the hallmark finding is absent

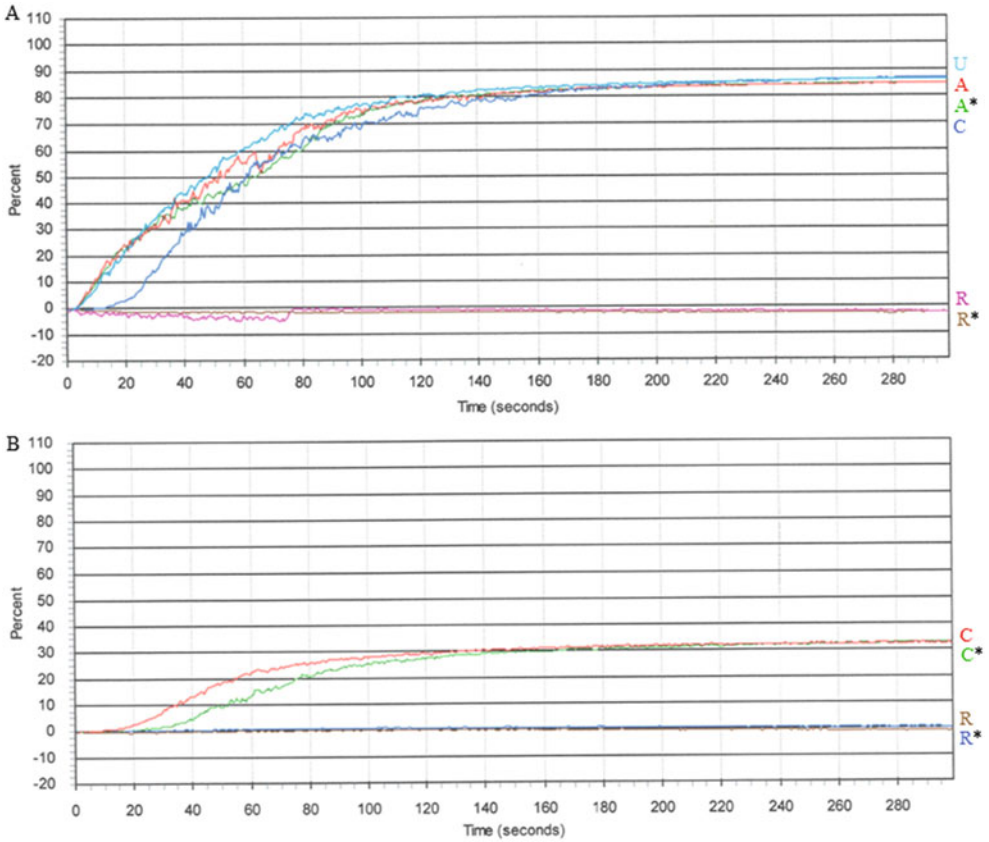

Fig. 5 Examples of aggregation abnormalities due to congenital Bernard Soulier syndrome. Panels show responses for subjects with different degrees of thrombocytopenia (respective sample platelet counts: A: 102 × 10^9/l; B: 17 × 10^9/l). In panel **A**, there is no agglutination with 0.5 (R*) or 1.25 (R) mg/ml ristocetin (R), whereas there is considerable aggregation with 5 µg/ml collagen (C), 2.5 (A*) and 5.0 (A) µM ADP and 1 µM thromboxane analogue U46619 (U). In panel **B**, there is no agglutination with ristocetin and the responses to both concentrations of Horm collagen (C*, 1.25 µg/ml; C, 5 µg/ml) appear reduced but is due to very low platelet count of the sample

agglutination with ristocetin (Fig. 5). In cases with more severe thrombocytopenia, the hallmark finding may be accompanied by reductions in maximal aggregation responses to other agonists, which are attributable to the thrombocytopenia (e.g., Fig. 5 lower panel).

Figure 3a shows an isolated abnormality in the aggregation response to ristocetin, tested at a concentration that agglutinates normal platelets (e.g., 1.25 mg/ml). In this case, the delayed agglutination with ristocetin is due to type 2M von Willebrand disease. Figure 3b illustrates a gain-of-function defect in the agglutination response to a low concentration of ristocetin (e.g., 0.5 mg/ml) in a subject with type 2B von Willebrand disease results in increased agglutination, followed by aggregation (<7 % aggregation is considered normal).

Figure 6 shows aggregation abnormalities that are typical of aspirin and non-steroidal anti-inflammatory drugs (NSAID) that inhibit platelet cyclooxygenase 1 (COX1) but can also result from inherited defects in COX1 and thromboxane synthase (Mustard et al. 1975; Cattaneo et al. 1982). Aggregation is markedly impaired or absent with arachidonic acid; however, the aggregation response to exogenous thromboxane (in this case, thromboxane analogue U46619) is completely normal. With NSAID-induced defects, or inherited, aspirin-like defects, aggregation is typically more impaired with low compared to high concentrations of collagen. Commonly, there is no secondary aggregation response to epinephrine. In some cases, ristocetin-induced platelet aggregation is also impaired. Responses to ADP are typically attenuated compared to responses off NSAIDs, but in many cases, the response to ADP falls within the reference interval for maximal aggregation.

Figure 7 shows aggregation responses to a number of agonists in a subject with impaired platelet function that is evident in both aggregation and secretion tests due to a *RUNX1* mutation. In this example, the responses to arachidonic acid, thromboxane analogue U46619, and the lower concentration of collagen are impaired and there is no secondary aggregation response to epinephrine. There is potential to confuse the findings with aspirin-like defects, which can be avoided by including thromboxane analogue in the panel.

Figure 8 illustrated impaired epinephrine aggregation responses in a subject with Quebec platelet disorder. In this condition, the typical finding is minimal aggregation, or absent secondary aggregation, with epinephrine in LTA assays with either native or platelet count adjusted samples.

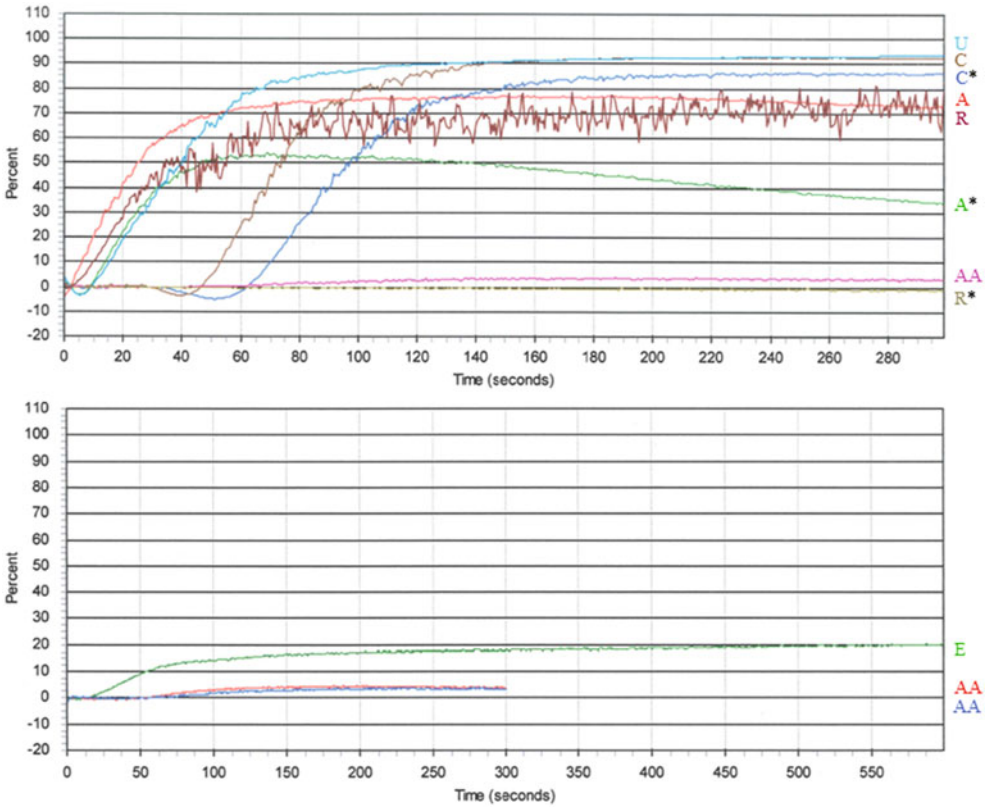

Fig. 6 Impaired platelet aggregation responses due to non-steroidal anti-inflammatory drugs that inhibit cyclooxygenase 1. Based on reference intervals (RI, shown after each agonist abbreviation) for maximal aggregation, there is markedly impaired aggregation with 1.6 mM arachidonic acid (AA >77 %) but a normal response to thromboxane analogue 1 μM U46619 (U >70 %). The maximal aggregation responses to 1.25 and 5 μg/ml Horm collagen (C* >51; C >85 %), 2.5 and 5 μM ADP (A* >24 %; A >43 %) and 0.5 mg/ml and 1.25 mg/ml ristocetin (R* <7 %; R >75 %) are normal. There is a small amount of deaggregation with both concentrations of ADP, which is not normal at the higher concentration. Lower panel: absent secondary aggregation with epinephrine (E) is a typical finding in addition to the reduced aggregation with arachidonic acid (AA)

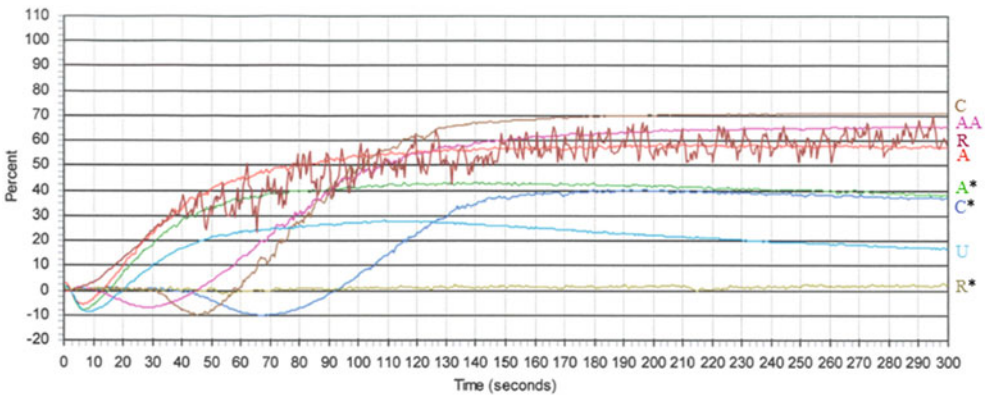

Fig. 7 Impaired platelet aggregation responses from a congenital platelet disorder caused by a *RUNX1* mutation. Based on maximal aggregation reference intervals, there is reduced aggregation with 1.6 mM arachidonic acid (AA >77 %), 1.25 and 5 μg/ml Horm collagen (C* >51; C >85 %), and reduced aggregation and some deaggregation with 1 μM thromboxane analogue U46619 (U >70 %). Responses to 2.5 and 5.0 μM ADP (A* >24 %; A >43 %) and 0.5 and 1.25 mg/ml ristocetin (R* <7 %; R >75 %) are normal

Primary aggregation can be reduced, as illustrated in this example.

Figure 9 shows an aggregation study of a subject with dense granule deficiency. The aggregation findings are typically normal in about one third of cases (as observed for this example), with other cases showing impairments with one or more agonists, and absent secondary responses to epinephrine. Typically, the aggregation responses to ADP are normal when there is an isolated dense granule deficiency, reflecting that adding exogenous ADP can overcome the functional consequences of having reduced platelet stores of ADP and other dense granule components. It can be challenging to distinguish platelet function disorders due to dense granule deficiency from defects that impair platelet secretion due to other causes unless dense granules, or their contents, are directly evaluated.

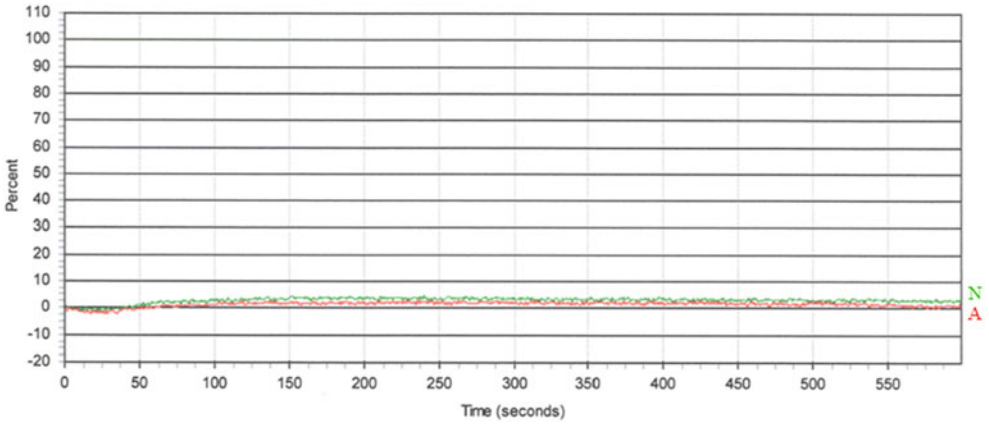

Fig. 8 Impaired aggregation with 6 μM epinephrine due to Quebec platelet disorder. The primary aggregation response to epinephrine is markedly reduced with both native (N) and platelet count adjusted (A) platelet rich plasma. Secondary aggregation is absent

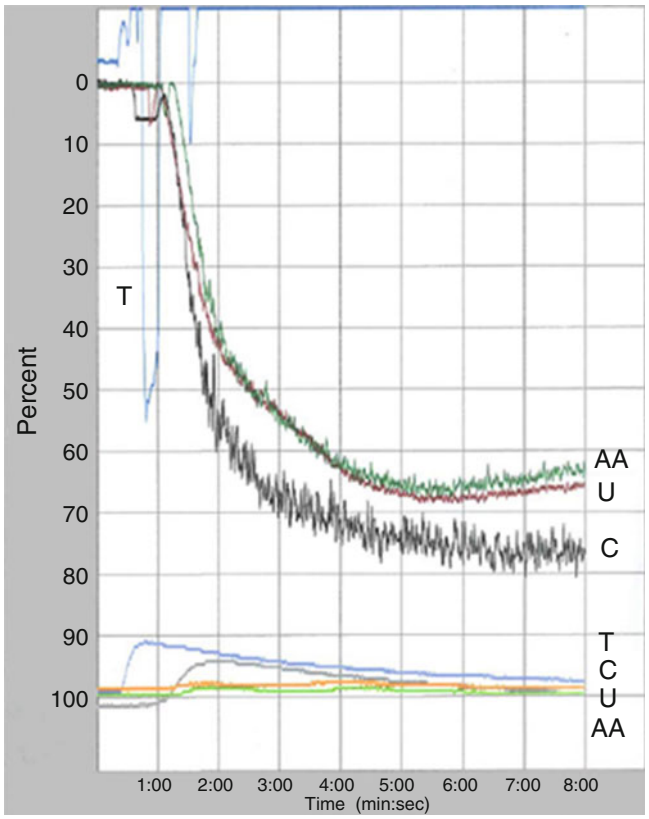

Fig. 9 Aggregation findings for a subject with dense granule deficiency. In this case, there is minimal ATP release (lower tracings) with the strong agonists thrombin (T) and collagen (C), and even less release with arachidonic acid (AA) and thromboxane analogue (U). The aggregation tracing with thrombin shows clotting of the sample

First steps in the clinical interpretation of aggregation findings

It is useful to consider guideline recommendations (Hayward et al. 2010), and the following list of questions, whenever aggregation studies are to be interpreted.

Were the responses for the responses for the quality control sample within reference ranges?

Was the subject thrombocytopenic? If yes, be cautious as a low platelet count affects aggregation responses to many agonists.

Is this a first or a repeat test? If it is a repeat test, the findings should be reviewed with comparison to previous results.

Did the subject have platelets of normal size or were they abnormally large (macrothrombocytopenic? giant?)

Was the sample normal in appearance or lipemic?

Were there any deviations from the standard operating procedure (e.g., was testing completed within 4 h?)

Was the full panel of agonists tested, and if any responses were impaired, was there sufficient sample to retest and confirm all findings outside the reference interval?

If the sample tested by aggregometry had a normal platelet count, consider:

Do any maximal aggregation findings fall below the laboratory's reference range for healthy controls (or above the reference range for the low concentration of ristocetin)? If yes, was there abnormal deaggregation with any of the agonists?

If all aggregation findings were within reference intervals, the test can be reported as non-diagnostic.

*Note: normal aggregation findings only exclude some platelet disorders as some conditions (*e.g.*, dense granule deficiency) are not always associated with*

(continued)

*impaired aggregation responses. Furthermore, some platelet disorders (*e.g.*, MHY9 related disorders) do not typically cause aggregation abnormalities.*

Guidelines have been published to standardize and improve the interpretation of aggregation tests as many diagnostic laboratories that assess platelet function by aggregometry have difficulty with interpreting the findings (Hayward et al. 2010, 2012). The authors approach to interpretation (which considers these guideline recommendations) is summarized in Table 2.

Evaluation of Aggregation Findings for Low Platelet Count Samples

Subjects who have mild thrombocytopenia often have platelet rich plasma platelet counts that are $\geq$ 200–250 $\times$ 10^9 platelet/l, including those with type 2B and platelet type

Table 2 Approach to interpreting aggregation abnormalities, based on the test findings

Aggregation finding	Interpretation
Aggregation is absent or markedly reduced with arachidonic acid, but the response to thromboxane analogue is normal. There may also be reduced aggregation with low concentrations of collagen, and absent secondary aggregation with epinephrine	The aggregation findings suggest an aspirin-like defect, which can be drug-induced or inherited. The drug history should be reviewed, with consideration of repeat testing when subject is not taking aspirin or non-steroidal anti-inflammatory drugs. *Note: If the testing did not include an evaluation of aggregation with thromboxane analogue, the interpretation needs to also state:* There are many other potential explanations for the aggregation abnormalities, which could reflect a congenital or acquired defect in platelet function. If drug-induced causes are excluded, further testing should be considered to evaluate for associated defects in dense granule release and/or dense granule numbers
Aggregation is present only with ristocetin. The extent of aggregation with ristocetin may be reduced	The aggregation findings suggest possible Glanzmann thrombasthenia, which can be inherited or acquired due to drugs, autoantibodies, and other causes. Repeat aggregation studies should be considered, along with flow cytometry studies to assess for $\alpha_{IIb}\beta_3$ deficiency
Aggregation is absent with high concentrations of ristocetin. Records show that the subject has normal von Willebrand factor levels. There may also be thrombocytopenia and very large platelets	The aggregation findings suggest Bernard Soulier syndrome, which can be inherited or acquired due to autoantibodies and other causes. Repeat aggregation studies should be considered, along with flow cytometry studies to assess for glycoprotein IbIXV deficiency
Aggregation is abnormally increased with low concentrations of ristocetin. If available, von Willebrand factor levels should be reviewed	There is abnormally increased aggregation with ristocetin, which suggests the possibility of type 2B or platelet type von Willebrand disease. Further testing should be considered, to exclude a false positive, with consideration of genetic studies if the finding is confirmed and/or associated with abnormalities in von Willebrand factor
Aggregation is impaired with a number of agonists and there is marked impairment in the response to ADP, with significant deaggregation	The marked impairment in ADP aggregation responses suggest the possibility of a defect involving the platelet ADP receptor, P2Y12, which can be congenital or acquired due to drugs. If the history excludes a drug-induced defect, repeat aggregation testing should be considered
Aggregation responses show other abnormalities with $\geq$2 agonists	The interpretation should first state which aggregation responses are impaired and which show deaggregation. The impaired aggregation responses to multiple agonists suggest a platelet function disorder. In this case, the abnormalities are not consistent with Glanzmann thrombasthenia, or a defect induced by aspirin, non-steroidal anti-inflammatory drugs or P2Y12 inhibitors. Further testing should be considered to confirm the findings and to assess for associated defects in dense granule release and/or dense granule numbers
Aggregation is abnormal with only 1 agonist, which is not ristocetin or collagen	The interpretation should state which response is abnormal. A single agonist abnormality is considered non-diagnostic and could represent a false-positive. Repeat aggregation studies should be considered, along with an assessment for defects in dense granule release and/or dense granule numbers

A number of defects have inherited and acquired causes so it is important that the interpretation of findings considers all possibilities. The recommendations in this table consider the North American guidelines (Hayward et al. 2010), and new information on the findings for different congenital platelet function disorders. If dense granule release is performed at the same time as aggregation studies, the interpretation should be modified to describe the results for both endpoints

von Willebrand disease. With some conditions, including Bernard Soulier syndrome, the thrombocytopenia is often much more severe. With some agonists, including arachidonic and thromboxane analogue U46619, minor reductions in sample platelet count can have profound effects on the aggregation response, whereas ristocetin can usually be tested, even if the platelet count of a sample is very low (see Fig. 5). There is more variability in aggregation responses when the sample platelet count is quite low (Hayward et al. 2008). Some laboratories use accumulated data for healthy control samples adjusted with platelet poor plasma to match the platelet count of thrombocytopenic patient samples to guide the test interpretation (Hayward et al. 2008) as was done for the case of Bernard Soulier syndrome shown in Fig. 5b. Laboratories should be prepared to accept aggregation requests for subjects with severe thrombocytopenia, although when the platelet count is very low, testing may not be possible with most agonists, except ristocetin. Failure to accept samples from individuals with thrombocytopenia can compromise the diagnosis of Bernard Soulier syndrome. In a recent quality assurance exercise, many laboratories that perform diagnostic LTA failed to recognize that the case in Fig. 5b was an example of Bernard Soulier syndrome (Hayward et al. 2012). Similar pitfalls likely exist in testing thrombocytopenic individuals for variant Glanzmann thrombasthenia (Nurden et al. 2011).

Pitfalls to Avoid in Aggregation Test Interpretation

Quality assurance exercises, to evaluate the interpretation of LTA, have provided important insights on common errors and pitfalls (Hayward et al. 2012). A number of common mistakes made by clinical laboratories are summarized below. Many of these mistakes could be avoided if laboratories followed guidelines and recommendations on how to interpret platelet aggregation responses (Hayward et al. 2010, 2012).

Common errors in the interpretation of aggregation findings

Failure to recognize normal variants (e.g., no secondary aggregation with epinephrine for aggregation studies performed with platelet count adjusted samples).

Failure to consider that the abnormalities could have congenital or acquired causes.

Failure to report the findings along with a proper reference interval, derived by nonparametric statistical approaches (most agonist responses are not normally distributed).

Failure to consider that use of a limited agonist panel may reduce detection of abnormalities, or the ability to distinguish probable false from true positives.

Failure to check if the patient is taking a potentially interfering substance.

Failure to consider the effect of a low platelet count on platelet aggregation responses.

Attributing an abnormality to a drug, without adequately considering the full range of potential causes, particularly when aggregation is reduced with arachidonic acid, as this can reflect a range of conditions that include secretion defects, with or without dense granule deficiency.

Concluding that a single agonist abnormality is significant when it often represents a false positive (exception: abnormalities that affect only collagen or ristocetin could have a biological explanation).

Concluding that aggregation abnormalities are due to a secretion defect or dense granule deficiency when neither secretion responses nor dense granule numbers were directly evaluated.

Reporting incorrect diagnoses.

Reporting that a finding is diagnostic of a platelet disorder when further testing is needed.

Failure to consider a less common explanation for an abnormality (e.g., platelet type von Willebrand disease when aggregation is increased with the low concentration of ristocetin).

Failure to recognize patterns for rare disorders, such as Glanzmann thrombasthenia and Bernard Soulier syndrome, and/or recommend further testing for these disorders.

Reporting an interpretation that is difficult for a clinician to understand.

Current Challenges in Diagnostic Aggregometry

Since the description of LTA by Born (1962) and O'Brien (1962), there have been only modest improvements in the instrumentation for performing aggregometry, which remains one of the most time-consuming, manual tests performed by coagulation laboratories. There has never

been a prospective study to determine if WBA is non-inferior to LTA, or if one of these methods is superior for diagnosing a bleeding disorder. Such studies are very much needed. Many of the guideline recommendations for aggregometry would benefit from research studies to test expert recommendations. Many laboratories report difficulties with procuring healthy control samples for ongoing quality monitoring of their aggregation assays and for establishing appropriate reference intervals for the test parameters. Few laboratories have assessed their method to validate if their procedure provides adequate detection of common and rare platelet function disorders. There is need for further research to address the value of aggregometry for bleeding disorder diagnosis, and to generate more evidence on appropriate protocols and procedures for diagnosing bleeding disorders using aggregometry. As there are increasing requirements in many developed countries for clinical laboratories to thoroughly validate all non-commercial, "laboratory developed" protocols and methods (which by its nature, includes aggregometry), this poses further challenges to adopting recommendations that lack published evidence. There are emerging complex reasons why platelet aggregometry responses are impaired in many common platelet function disorders. Accordingly, aggregation phenotype/genotype relationships need to be explored to a greater depth in order to develop more evidence-based approaches to the evaluation of platelet function disorders.

Take Home Messages

- Platelet aggregometry is a fifty-year-old method that remains important for assessing platelet function for diagnostic and research purposes.
- Laboratories that perform platelet aggregation testing should be aware of recent guidelines on how to prepare and process samples, test aggregation responses, and how to analyze, interpret, and report the findings.
- Maximal aggregation responses are very important for interpreting aggregation findings, which needs to consider which agonist response(s) are abnormal, and the potential congenital and acquired defects that could cause the observed abnormalities, including preanalytical and analytical errors and false positive findings.
- There is now good evidence that platelet aggregometry is useful for bleeding disorder diagnosis, if performed by validated methods, with appropriate quality controls to verify assay performance, and reference intervals to guide the test interpretation.
- At the present time, LTA remains the best validated aggregometry procedure. Comparison studies would be useful to determine if WBA is non-inferior to LTA for the purpose of diagnosing a bleeding disorder.

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Platelet Secretion

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Abstract

In this chapter, we summarise existing methods for the measurement of platelet δ, α-granule and lysosome secretion, discussing the advantages and disadvantages of their application in both the clinic and research laboratories.

Introduction

Platelet Granules and Contents

Under physiological conditions, platelets are relatively inert circulatory cells primarily engaged in 'surveying' the vasculature in search of damage to the endothelium. A range of extracellular matrix structures are revealed upon endothelial damage that can initiate a rapidly propagating signalling cascade in platelets, which eventually leads to the formation of a stable clot, or thrombus, at the site of vascular injury. In addition, platelet activation leads to the secretion of 300+ bioactive molecules from releasable granules. Platelet secretion remains incompletely understood (Golebiewska and Poole 2013), but it is becoming increasingly clear that it plays a role in physiological and pathological processes beyond the 'traditional' thrombotic/haemostatic scenarios (Golebiewska and Poole 2015).

The function of each of the platelet secretory granules (principally dense granule, α-granule and lysosome) is defined by their contents. The diversity of granule cargoes and specific examples of each granule contents and function are illustrated in Table 1.

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Utility of Platelet Secretion Testing

Inherited Storage Pool Disorders

The synthesis and packaging of granules are thought to occur mainly at the early megakaryocyte stage (Machlus and Italiano 2013), but little is known about exact mechanisms of granules sorting and trafficking to their final destination in platelets (Ambrosio et al. 2012). Defective granule synthesis results in storage pool disorders (SPD), where platelets are usually devoid of either dense (δ) granules (Hermansky-Pudlak syndrome, HPS, and Chediak-Higashi syndrome, CHS (Masliah-Planchon et al. 2013)), or alpha (α) granules (grey platelet syndrome, GPS). Bleeding manifestations among SPD patients include spontaneous bruising, epistaxis, menorrhagia and prolonged oozing after trauma or minor surgery such as a tooth extraction (Nurden et al. 2012). Storage pool disorders are one of the most prevalent inherited platelet disorders, with some estimates attributing almost 90 % of platelet disorders to defects in secretion (Mumford et al. 2015). Unlike in cases of severe inherited platelet defects such as Glanzmann's thrombasthenia or Bernard-Soulier syndrome, in which platelets have a profound aggregation defect and diagnosis can be confirmed by measurement of surface glycoproteins ($\alpha_{IIb}\beta_3$ and GPIb-V-IX, respectively), diagnosis of SPD requires a more complex array of tests, and it is likely that a high proportion of SPD patients go undiagnosed. Recently, the International Society on Thrombosis and Haemostasis (ISTH) included α and δ-granule secretion testing as first-line tests recommended for diagnosis of inherited platelet disorders, to rule in or out SPD (Gresele and Subcommittee on Platelet 2015).

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Table 1 Summary of some of the platelet granule cargoes and their functional classification

Granule type	Cargo	Examples	Function
Dense	Small molecules	ADP, ATP, 5-HT polyphosphates	Platelet agonists coagulation cofactor
Alpha	Proteins	P-selectin, vWF	Adhesion molecules
		PF-4, RANTES, PDGF	Chemo/cytokines
		VEGF, SDF-1α	Pro-angiogenic factors
		Endostatin, angiostatin	Anti-angiogenic factors
		Factor V, factor XII Amyloid precursor protein	Coagulation factors Integral membrane protein
Lysosomes	Proteins	β-Hexosaminidase, lactate dehydrogenase, metalloproteinases	Enzymes (clot remodelling)
		LAMP-3	Membrane proteins

Other Secretion Defects

SPD can also develop in response to a number of external factors ('acquired' SPD)—limited reports implicate clinical conditions including idiopathic myelofibrosis (Mouly et al. 2000), lupus (Pareti et al. 1980; Weiss et al. 1980) and renal failure (Soslau et al. 1990) as well as clinical interventions such as tamoxifen treatment (Nayak and Schmaier 2012) and bone marrow transplantation (Sakashita et al. 2001) in causing SPD. While the aetiology of acquired SPD remains unclear, one of the mechanisms may be exposure of platelets to activators such as damaged endothelium, thrombin and immune complexes leading to degranulation and circulation of 'exhausted' platelets (Pareti et al. 1980; O'Brien 1978). Acquired SPD is likely more prevalent than hereditary SPD and can lead to exacerbation of the underlying condition. Platelet secretion testing in those conditions known to be associated with platelet 'exhaustion' may therefore aid diagnosis and treatment. SPD or secretion defects may also arise in platelets during preparation for platelet transfusions (Washitani et al. 1988), in which case secretion testing may be a useful 'quality control' measure for this application (Deckmyn and Feys 2013).

Secretion testing of platelets may also aid in the diagnosis of a range of other systemic disorders associated with membrane trafficking. For instance, familial haemophagocytic lymphohistiocytosis (FHL) subtypes 3, 4 and 5 result from genetic mutation/deletion of Munc13-4, Syntaxin 11 and Munc18-2, respectively, all of which are essential in the secretory machinery of platelets (Golebiewska and Poole 2013; Ye et al. 2012; Al Hawas et al. 2012).

At the other end of the spectrum, an increase in platelet secretion can indicate generalised hyperactivity of platelets, which in turn may lead to increased cardiovascular risk. For example, platelets of diabetic patients have been shown to have increased ATP content and ATP secretion (Guo et al. 2009; Michno et al. 2007). Monitoring platelet secretion in those patients may predict increased risk of cardiovascular disease as comorbidity with diabetes. Equally importantly, the platelet serotonin release assay is 'virtually diagnostic of heparin-induced thrombocytopenia' (Warkentin et al. 2015) when used in appropriate clinical context, allowing for management of this potentially life-threatening adverse reaction to routine heparin treatment (Sheridan et al. 1986).

Platelets as Biomarkers

The platelet secretome is of particular interest in the rapidly expanding array of functions for platelets beyond 'traditional' haemostasis and thrombosis. For example, platelets are known to express several components of the pathway associated with Alzheimer's disease and thus have been used in the search for biomarkers of the disease (Veitinger et al. 2014; Gowert et al. 2014). Platelets are the most prominent peripheral source of APP (amyloid precursor protein), and it has been shown that secretion of a variant sAPP-beta but not sAPP-alpha is significantly increased in Alzheimer's patients (Evin and Li 2012). It is also specifically secreted upon activation of platelets, after cleavage by beta-site APP-cleaving enzyme (BACE1), a secretase associated with Alzheimer's development (Evin and Li 2012; Marksteiner and Humpel 2013). There is therefore a potential for using platelets as a non-invasive source of biomarkers for early Alzheimer's diagnosis.

There is also great potential for platelets as biomarkers in cancer. Platelets have been shown to sequester proteins in the bloodstream, and the concentration of pro-angiogenic markers in platelets, but not in plasma, is increased in the presence of the tumour (Almog and Klement 2010). Platelet factor 4 (PF-4) in particular has been shown to be a marker of early tumour growth in animal models (Cervi et al. 2008). Therefore, measuring platelet secretion can be an indirect means of measuring angiogenic activity in the body.

Methods for Platelet Secretion Measurements

Platelet Preparation Considerations

As with all platelet function studies, it is important to consider how platelets are prepared and/or isolated for assessment of secretion (Cazenave et al. 2004). Whole-blood

(WB) preparations are closest to physiological conditions but also contain a proportionately large volume of anticoagulant and must be constantly mixed by the use of a roller before assessment of secretion. It is for this reason that whole-blood impedance-based lumi-aggregometry is rarely performed and not validated for clinical assessment (McGlasson and Fritsma 2009; Gresele et al. 2014). Platelet-rich plasma (PRP) preparations usually require the use of a centrifuge to isolate platelets together with plasma proteins, which is beneficial to multiple assays due to the lack of red blood cells. However, both PRP and whole-blood preparations are not ideal for ELISAs, microarrays or imaging due to the presence of other blood components—which make interpreting results much harder.

Operators may consider opting for washed platelet (WP) preparations rather than PRP or whole blood in certain instances. These preparations require two separate centrifugation steps, before the platelets are resuspended in HEPES-Tyrode's solution, resulting in a pure platelet suspension with minimal contamination of other blood cells and plasma proteins. Like other platelet preparations, washed platelet preparations require the addition of a specific combination of anticoagulants to prevent platelet activation due to handling, something which should be carefully considered. For example, apyrase, which is sometimes used to minimise P2Y1/12-mediated activation by scavenging free ADP during platelet preparation, may, if used at too high a concentration, block the effect of ADP used as an agonist in the assay. While addition of inhibitors can limit activation, the sensitivity of the response of washed platelets still declines rapidly, and therefore this preparation is most suitable for immediate analysis (Truss et al. 2009). Additionally, washed platelets are highly sensitive to ion concentration and pH of the preparation, and care must be taken to ensure an optimal response is achieved.

Overall, choosing the right preparation will depend upon the type and aims of the assay to be performed (Table 2). It may also be beneficial, where possible, to test different preparations in parallel, e.g. when testing the efficacy of novel anti-platelet therapies, as the presence of plasma proteins can influence drug receptor binding and give different results in whole blood, PRP and washed platelet preparations (Sun et al. 2001; Chlopicki et al. 2012).

Bioluminescent Assays

In clinical settings, lumi-aggregometry is the most commonly used technique to analyse platelet secretion (Gresele et al. 2014) and is one of the primary analyses for the evaluation of any suspected platelet function disorder (Harrison et al. 2011; Lambert 2011; Brass 2010). In this procedure firefly luciferin-luciferase is used to produce a light output proportional to ATP released into the extracellular medium by stimulated platelets (Feinman et al. 1977; Heath 2004). Light output is detected by a photometer, calibrated against a standard of known ATP concentration. It is also possible to measure ADP and AMP release using a modification to this approach that sequentially phosphorylates these nucleotides up to ATP, allowing reaction with luciferin-luciferase. So, addition of pyruvate kinase and phosphoenolpyruvate allows the measurement of ADP,

Table 2 An overview of the main techniques used to assess different types of platelet secretion and the appropriate platelet preparation to be used with each method

	Granule	Marker	Preparation
Impedance-based lumi-aggregometry	δ	ATP	WB
Light transmission lumi-aggregometry	δ	ATP	PRP, WP
Flow cytometry	α	P-selectin, fibrinogen	WB, PRP, WP
	δ	Mepacrine	WB, PRP, WP
	Lys	CD63	WB, PRP, WP
14C/3H 5-HT release assay	δ	ATP	WP
HPLC/LC-MS	δ	ATP, 5-HT	WP
ELISA	α	e.g., P-selectin, PF-4, β-TG	WP
	δ	5-HT	WP
Microarray	α	P-selectin, PF-4, APP, pro-/anti-angiogenic profile, etc.	WP
	δ	5-HT	WP
Lysosomal secretion assays	Lys	β-Hex, LDH	WP
Standard TEM	α, δ	–	WP
Whole mount TEM	δ	–	WP, PRP
Immunofluorescence	α	P-selectin, PF-4	WP
	δ	5-HT	WP

WB whole blood, *PRP* platelet-rich plasma, *WP* washed platelets

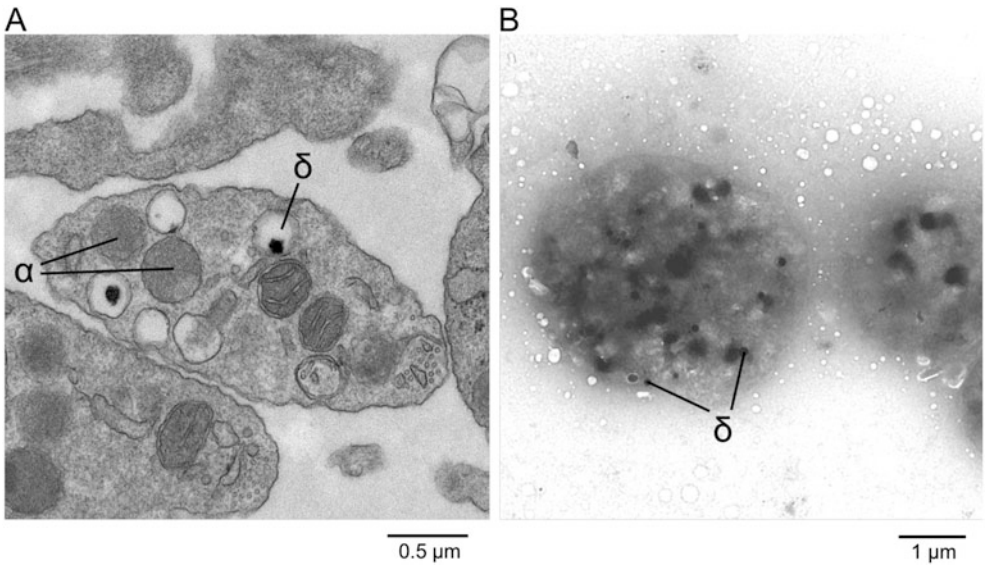

Fig. 1 Images of human platelets generated with TEM using both standard (**a**) and whole mount (**b**) preparations. Standard TEM allows for identification of both δ- and α-granules, whereas it is only possible to reliably identify δ-granules in whole mount preparations due to the lower-resolution achieved. Images courtesy of Dr. Ed Brown, University of Bristol, UK and Dr. Rob Goggs, Cornell University, USA

with a further addition of adenylate kinase and cytidine 5′-triphosphate allowing for measurement of AMP release (Heath 2004).

$$\text{ATP} + \text{D-luciferin} + \text{O}_2 \rightarrow \text{AMP} + \text{PP}_\text{i} + \text{oxyluciferin} + \text{CO}_2 + \text{light}$$

$$\text{ADP} + \text{PEP} \rightarrow \text{ATP} + \text{pyruvate}$$

$$\text{AMP} + \text{CTP} \rightarrow \text{ADP} + \text{CDP}$$

As releasable nucleotides are stored in δ-granules (Fig. 1), this technique is classed as a direct measure of δ-granule secretion and is therefore used clinically to detect defects in this type of secretion (Harrison et al. 2011). Historically, lumi-aggregometry has been extremely important to the field of platelet research, allowing for the study of the molecular mechanisms and signalling pathways associated with δ-granule secretion (Golebiewska and Poole 2015). The methodology itself also allows simultaneous aggregation traces to be recorded which can be used clinically to aid the identification of platelet defects.

Lumi-aggregometry may also aid in the discrimination between storage pool defects such as Hermansky-Pudlak syndrome (HPS) (Li et al. 2003; DePinho and Kaplan 1985) and secretory machinery defects such as familial hemophagocytic lymphohistiocytosis type 3 (Nakamura et al. 2015). Calculation of released ATP/ADP ratios may indicate the presence of a storage pool defect if the ratio is >2, because in healthy subjects ADP is released in greater concentrations than ATP with typical physiological ratios being in the 0.65–0.78 range (Holmsen and Weiss 1972; Weiss et al. 1979). However, there are limitations to consider when performing lumi-aggregometry, such as the high rates of intra- and interindividual variability. Patient platelet responses should therefore be tested against a healthy control sample with a panel of agonists over several days (Pai et al. 2011; Lotta et al. 2013). Between laboratories it is also not uncommon to see large variability in ATP secretion results (Moffat et al. 2005), which can make interpretation of multisite studies challenging. Despite these variabilities in lumi-aggregometry for the measurement of platelet δ-granule secretion, its relative ease of use, low price of consumables and rapid acquisition of results mean that it will continue to be used as a valuable research and diagnostic tool. An alternative approach is the use of whole-blood impedance aggregometry coupled with bioluminescence, which in a recent study was shown to be more sensitive to changes in platelet function than optical lumi-aggregometry (McGlasson and Fritsma 2009). This technique is also less technically demanding, particularly since no platelet isolation steps are required for preparation of samples, and is therefore a potentially attractive clinical diagnostic approach.

Flow Cytometry

Clinically, flow cytometry is routinely used to measure surface expression of glycoproteins on platelets in suspected cases of Glanzmann's thrombasthenia and Bernard-Soulier syndrome, but is less often used to measure platelet secretion (Gresele et al. 2014; Nurden and Caen 1974; Shulman and Karpatkin 1980). In the research laboratory, however, it is a commonly used approach for measurement particularly of α-granule secretion. Upon platelet activation and subsequent secretion, integral granule membrane proteins become incorporated into the plasma membrane, upon fusion of granules with the membrane, and therefore become

surface-exposed (Rosa et al. 1987). The plasma membrane bound proteins can then be detected with fluorescently-labelled primary or secondary antibodies and an appropriately calibrated flow cytometer which identifies the platelet sub-population by forward and side scatter values, allowing for the measurement of platelet secretion (Johnston et al. 1987; Tschoepe et al. 1990). Typically the expression of the α-granule marker P-selectin (CD62P) is used as a measure of α-granule secretion (Michelson et al. 2000), although it must be noted that low levels of P-selectin are also present in δ-granules (Israels et al. 1992). A recent study has shown CD40L (CD154) to be present in α-granules (Charafeddine et al. 2012), which could therefore also be used as an α-granule secretion marker—with antibodies readily available for such purposes (Furman et al. 2004). Some studies have however shown the release of CD40L to be slower than other α-granule content, suggesting its measurement may not be properly reflected α-granule secretion, or that it may be localised to sites other than α-granules (Henn et al. 2001; Jin et al. 2001; Nannizzi-Alaimo et al. 2003). CD63 (LAMP-3) expression is regularly used as a marker of both δ-granule and lysosomal secretion due to the presence of this protein in the membrane of both of these secretory granules (Nishibori et al. 1993). Ideally, platelets would be stimulated with a panel of agonists at multiple concentrations (including a vehicle and unstained control) in parallel with a healthy control sample, before analysis by flow cytometry.

Flow cytometry as a technique for analysing both platelet function and secretion has several advantages. In a recent study, nearly 60 % of haematology laboratories surveyed had access to a flow cytometer—making it one of the most accessible techniques in the clinic (Gresele et al. 2014). If access to a flow cytometer is not available, off-site analysis of fixed preparations is also possible and has been successfully performed during multisite testing of anti-platelet agents (Frelinger et al. 2011; Michelson et al. 2007). Fixation of whole blood for flow cytometric analysis at a remote site can be made with proprietary fixative solutions, such as PAMfix (Platelet Solutions, Nottingham, UK), which enables flow cytometric platelet analysis to be undertaken remotely (Dovlatova 2015). Another major advantage of flow cytometry is that relatively few platelets are required for analysis, making it a technique appealing to situations involving both paediatric and thrombocytopenic patients. The possibility of using multiple fluorophores (with appropriate compensation controls) also allows for the monitoring of multiple aspects of platelet function simultaneously, further reducing the number of platelets needed for assessment.

Flow cytometry can also be used to measure platelet δ-granule storage and secretion using a mepacrine (quinacrine) uptake and release assay (Wall et al. 1995; Gordon et al. 1995; Ramstrom et al. 1999). Samples of platelets are incubated with the fluorescent marker mepacrine, which is rapidly taken up into δ-granules (Da Prada and Pletscher 1975; Ambrosio et al. 2012), before analysis of uptake by flow cytometry against a control sample. Mepacrine release is then monitored after platelet stimulation, allowing for calculation of the difference between mepacrine-stained platelets before and after stimulation—quantifying δ-granule secretion. It is therefore suggested that this method would be able to discriminate between δ-granule storage pool defects and secretory machinery defects. However, further validation work will be needed to adopt this approach, since only a handful of groups have currently worked with this technique. The lack of studies could be due to the drawbacks associated with using mepacrine—including rapid loss of fluorescence, retention of mepacrine in the cytoplasm and a semi-quantitative end point (Mumford et al. 2015). It is also possible to use fluorescently labelled anti-5-HT antibodies to detect cargo in δ-granules, which has the potential for assessing storage pool disorders. However, this approach has not been widely reported (Maurer-Spurej et al. 2002) and suffers from the fact that the platelets need to be fixed and permeabilised as part of the preparation steps, introducing potential additional points of variability.

Flow cytometry is therefore a powerful approach for the assessment of platelet secretion in both clinical and research settings. It is a complex technique that requires skilled operators, and the initial cost of purchase and maintenance of the machine is expensive. However, reagent costs are generally low, and there are several smaller and cheaper 'bench top' flow cytometers now available, with parameters more tailored to platelet studies (Masters and Harrison 2014), which will make this technique increasingly accessible to investigators and clinics in the future (Tung et al. 2007).

5-HT Release Assay

Radioisotope Based Assay

Long considered to be the 'gold standard' in measuring δ-granule secretion, the [^{14}C]/[^{3}H] 5-HT release assay was one of the first assays developed to measure platelet secretion and is still used as a diagnostic tool over three decades later (Quiroga et al. 2009; Warkentin 2011). The assay itself is based on the exploitation of 5-HT uptake and release using either ^{14}C-5-HT or ^{3}H-5-HT. A small quantity of radiolabelled 5-HT is incubated with the platelet suspension, which is rapidly taken up into the platelet cytoplasm via the ligand gated ion channel SERT and subsequently translocated into δ-granules via the vesicular monoamine

transporter VMAT2 (Jedlitschky et al. 2012). Platelets are then stimulated with an appropriate agonist (or combination of agonists) causing degranulation and the release of radiolabelled 5-HT into the medium. A scintillation counter is used to determine the amount of released radiolabelled 5-HT, with the final value expressed as a fraction of the total 5-HT content in the platelets.

Overall this assay is fast and relatively cheap—with a recently published study highlighting the reproducibility of the technique (Quiroga et al. 2009). It also benefits from the ability to simultaneously record light transmission aggregometry, which can be used in conjunction with secretion data to evaluate potential platelet defects. Used alongside lumi-aggregometry, the [^{14}C]/[^{3}H] 5-HT release assay also allows operators to deduce if platelet defects are due to δ-granule biogenesis/loading or secretory machinery defects. However, due to its use of radio-isotopes, this assay has fallen out of favour with many laboratories, with only 16 % of ISTH surveyed laboratories now using this method (Gresele et al. 2014). It is also somewhat more laborious and time-consuming, requiring additional pieces of equipment, than luminometric approaches to δ-granule secretion.

Other Approaches

The use of *o*-phthalaldehyde provides another attractive radiolabel-free alternative to [^{14}C]/[^{3}H] 5-HT, which forms a fluorophore with 5-HT in situ (Drummond and Gordon 1974; Holmsen and Dangelmaier 1989). Platelets can then be stimulated with agonists leading to changes in fluorescence, signifying δ-granule secretion, being measured by a plate reader or similar fluorometer (Jedlitschky et al. 2010; Cattaneo et al. 2000).

Recently, a technique capable of measuring real-time 5-HT release has been described based on the electroactive nature of the endogenous serotonin molecules. It uses fast scan cyclic voltammetry, through carbon-fibre microelectrodes, but because of the specialist nature of the equipment required, it is not yet a first choice for routine analysis of platelet function in the clinic (Ge et al. 2011). High-performance liquid chromatography (HPLC) and ELISA-based approaches are also being investigated (see below). The drive to develop new 5-HT detection methods clearly supports its widely accepted clinical utility (Warkentin et al. 2015).

Liquid Chromatography and Mass Spectrometry

Chromatography techniques are powerful approaches to the separation and identification of molecules from complex mixed solutions. They are much more accessible to both researchers and clinicians than previously, largely due to the movement from gas to liquid chromatography-based systems (Adaway et al. 2015). Tandem mass spectral detection continues also to develop rapidly, and systems nowadays are remarkably sensitive as well as being able to deal with complex samples (Seger 2012; Wu and French 2013). They are therefore versatile, but specialist, assays developed to monitor platelet secretion in some haematology and research laboratories (Gresele et al. 2014).

High-performance liquid chromatography (HPLC) has been used to evaluate the storage and release of nucleotide pools in platelets, allowing for the specific separation and detection of adenine, guanine and cyclic nucleotides, with sensitivities down to picomolar concentrations using ultraviolet detectors (D'Souza and Glueck 1977; Leoncini et al. 1987). More recent studies have shown effective assessment of stored and released 5-HT in platelets with the use of HPLC and amperometric (Kumar et al. 1990; Flachaire et al. 1990), fluorescent (Anderson et al. 1992; Sa et al. 2012) and mass spectrometry (Torfs et al. 2012) methods of detection. Due to the highly sensitive measurement capabilities of LC-MS, it has also been suggested that it could be used as a reference method for other techniques (Mumford et al. 2015) and potentially be optimised to measure α-granule and lysosomal secretion.

Although chromatography techniques offer highly sensitive and precise ways to quantify total and secreted concentrations of molecules in platelets, the initial cost and maintenance of LC-MS instruments are very high. The high complexity of the instrumentation usually requires a dedicated operator to direct initial assay development and run samples. The 'static' nature of the assays also means that LC-MS does not readily provide information about the kinetics of platelet secretion (Anderson et al. 1992). For these reasons, its adoption is currently small, but the strength of the approach, coupled with proteomics analytics, means that it could be used to provide large-scale information on secreted products from platelets (Clutterbuck et al. 2011)—making it a strong candidate for advancing platelet 'secretomics' research, with wider-scale clinical applicability in the future.

Immunodetection Techniques

ELISAs

Radioimmunoassays have classically been used to measure concentrations of specific molecules present in platelet preparations or plasma (Kaplan et al. 1978), but have largely been replaced with ELISAs in both research and clinical settings. Studies have shown successful measurement of α-granule secretion using PF4, β-TG and PDGF as markers (Takahashi et al. 1988; Schraw and Whiteheart 2005; Harrison et al. 1994), with δ-granule secretion being

detected with the use of 5-HT as a marker, although the latter was shown to be inferior to an LC-MS method of detection (Torfs et al. 2012). As well as now being widely available commercially, ELISAs also benefit from being relatively easy to perform with all reactions taking place in the same 96-well (or 384-well) assay plate.

Performing an ELISA requires antibodies (or antigens, if antibodies are to be measured in the test sample) to be immobilised to the assay plate before incubation with test samples, which allows complexes to form between the target protein and the immobilised antibodies (Gan and Patel 2013). The test sample is then washed out of the well before the addition of an antibody to detect the bound target protein. This antibody is then itself detected by the use of a secondary enzyme-conjugated antibody, before the addition of a chromogenic substrate for the enzyme which yields a visible colour change or fluorescence—indicating the presence of the target protein. This colour change/fluorescence can then be measured using an appropriate plate reader.

The quality of the output from ELISAs relies upon the binding activity and specificity of the antibody. Naturally this can be a drawback to the technique, with poor performing antibodies leading to both false positives and negatives. Operators must also carefully consider the specificity of expression of target molecules for accurate results.

Although ELISAs are used clinically for the diagnosis of numerous pathologies, including platelet disorders such as heparin-induced thrombocytopenia (Francis 2004), adoption for the measurement of platelet secretion is low. Despite availability of commercial 5-HT ELISA kits, only 1/207 laboratories surveyed by ISTH used it to assess δ-granule content, with no respondents using ELISAs to measure α-granule content or release (Gresele et al. 2014). It is assumed that lack of adoption can be attributed to variability of results and the currently high cost of commercially available assay kits.

Microarrays

Based on the same principle of antigen detection as ELISAs, antibody microarrays offer another method for measuring protein levels in complex samples such as platelet releasates (Wingren and Borrebaeck 2009; Angenendt 2005; Coppinger et al. 2007). This technology works around the ambient analyte model of Ekins and colleagues (Ekins and Chu 1999), where 'microspot' assays which rely on the immobilisation of antibodies (or antigens) on a few square microns should, in principle, be able to detect analytes with greater sensitivity when compared with macroscopic immunoassays. Although much of the methodology and principles of antibody microarrays overlap with ELISAs, they benefit from the significantly smaller sample volumes needed alongside the ability to multiplex multiple antibody probes onto one plate, with 'mega-dense' nanoarrays allowing for up to 10,000 probes to be used (Wingren and Borrebaeck 2007). Detection is achieved with either labelling techniques such as fluorescent dyes and enzyme conjugations, or with label-free techniques such as electrochemical readouts. Although the use of antibody arrays in other fields of medical sciences is now routine, studies into platelet secretion with these techniques have so far been limited (Coppinger et al. 2007).

As the use of immunodetection techniques allows for the quantification of the abundance of specific analytes in platelet releasates, they have the potential to be used in the diagnosis of other pathologies which may indirectly affect the content of platelet granules. In the future, with development of cheaper, more reproducible ELISA- and microarray-based assays of platelet secretion, the potential of 'platelets as biomarkers' can be realised.

β-Hexosaminidase and Lactate Dehydrogenase Activity Assays

Although the physiological role of lysosomal secretion in platelets has not been completely elucidated (Polasek 1989), their release can be altered in disease states and potentially contribute to thrombotic processes and complications (Emiliani et al. 2006). Measurement of lysosomal secretion is achieved with the use of simple colorimetric assays performed in 96-well assay plates which rely on the activity of enzymes secreted from lysosomes in platelets. Typically platelets are stimulated with the desired agonists before centrifugation and transfer of the supernatant to wells of an assay plate. A chromogenic enzyme substrate is then added to the supernatant which causes a colorimetric change that can be measured with the use of a plate reader. In these assays β-hexosaminidase is commonly measured with 4-methyl-umbellyferyl-*N*-acetyl-β-D-glucosaminide, whereas lactate dehydrogenase is measured with a combination of NADH and sodium pyruvate (Rodriguez et al. 1997).

As a whole these assays are very cheap to perform, with reagents available separately or as complete kits from large suppliers. As these assays only measure lysosomal secretion, they present an advantage over the use of flow cytometry, where the use of CD63 fluorescent antibodies detect both δ-granule and lysosomal secretion. They are also readily quantifiable and may provide more quantitative data than flow cytometry. However, because of our lack of understanding of the role of lysosomal secretion, there is currently no clear clinical need for this assay, and this technique is therefore currently reserved for dedicated platelet research laboratories. This was reflected in a recent ISTH survey, with none of the 207 participating laboratories using lysosomal secretion assays to investigate platelet function (Gresele et al. 2014).

Imaging Techniques

Standard TEM

Transmission electron microscopy (TEM) was initially developed as a research tool to observe inorganic materials at ~1000 times greater resolution than could be seen with a light microscope based on the use of electrons as opposed to light waves. With the use of appropriate fixatives, this method was then applied to biological tissues, allowing for the study of the ultrastructure of cells and eventually platelets (Glauert 1979; Wyffels 2001; Clauser and Cramer-Borde 2009). The subsequent widespread adoption of TEM techniques has since given tremendous insights into platelet structure, function and pathological roles. The ability to visualise membranes, secretory granules and the platelet cytoskeleton has also allowed for the use of TEM as a diagnostic tool in conjunction with the previously described functional assays. However, due to the often slow-turn around and specialist workload associated with preparing platelets for TEM, this technique is frequently reserved for special cases where the use of traditional functional assays for the evaluation of platelet secretion disorders alone may not be sufficient.

The observation of abnormalities in both the quantity and appearance of secretory granules is used in the validation of multiple secretory defects such as granule biogenesis and storage diseases. In normal platelets, α-granules are the most numerous and appear as dark electron dense structures with marginally lighter peripheral zones encircled by a visible membrane. In both grey platelet syndrome (GPS) and Paris-Trousseau/Jacobsen syndrome, α-granules appear enlarged (Raccuglia 1971), whereas there are no obvious disparities in Quebec platelet disorder (Hayward et al. 1997). Further investigation with labelling or staining techniques can then be used to identify these specific disorders, with Wright stain marking α-granules as grey in GPS (Raccuglia 1971). The number of δ-granules in platelets is far fewer than that of α-granules (a mean of 5 per platelet), and they are clearly identified due to their morphology—an electron dense core surrounded by a visible 'halo' (Clauser and Cramer-Borde 2009). In δ-storage pool diseases such as HPS and CHS, the number of δ-granules are markedly reduced or completely absent, with the added presence of giant intracytoplasmic lysosomes in the latter (Hardisty and Hutton 1967; Rendu et al. 1983; White 1978).

It is important to consider how platelets are prepared for study by TEM due to the range of methods available and the benefits of each. The most common technique, which also allows for the generation of the highest resolution images, involves treating the platelets with a series of fixative steps before being dehydrated and subsequently embedded in a plastic resin (Clauser and Cramer-Borde 2009). This plastic block is then cut into ultrathin (700 Å) sections using an ultramicrotome before contrast enhancement with heavy metal staining. This method allows the visualisation of the complete platelet morphology, including secretory granules (Fig. 2a). However, operators should be aware that due to the limited number of δ-granules present in platelets, it is possible that cross-section images do not capture them, and therefore numerous sections should be imaged (White et al. 2007).

Whole Mount TEM

For this reason, a whole mount platelet preparation is usually favoured over embedding techniques, and this approach also offers the advantage of relative simplicity and rapid turnaround. In this preparation, small drops of PRP are placed on formvar-coated, carbon-stabilised (or similar) grids which are rinsed briefly with drops of

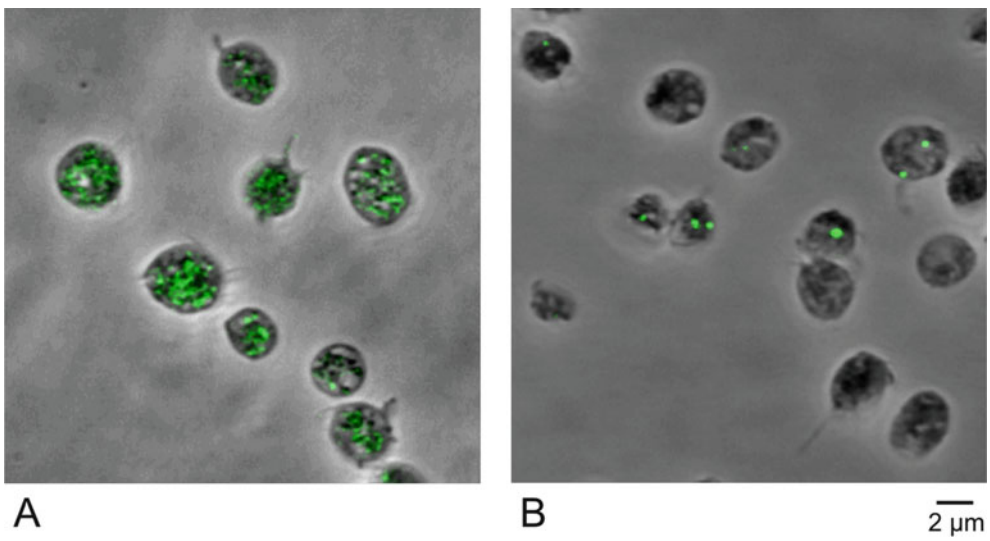

Fig. 2 Human platelets fixed and labelled with fluorophore-conjugated antibodies targeting P-selectin (**a**) and 5-HT (**b**), allowing for the visualisation of α- and δ-granules respectively. Images courtesy of Dr Chris Williams, University of Bristol, UK

distilled water (Witkop et al. 1987). Residual moisture is then air-dried from the grids before imaging via TEM. The images produced with this technique are of a lower resolution when compared directly with embedding techniques, but allow for direct enumeration and visualisation of δ-granules (Fig. 2b). It must however be mentioned that due to the lower resolution, the identification of α-granules can prove difficult, making whole mount preparations unsuitable for the analysis of α-granule defects.

With minor modifications to protocols, TEM also allows for the labelling of proteins at the ultrastructure level with the use of immunogold reagents in both embedded and whole mount preparations (Skepper and Powell 2008; Engelhardt et al. 2007). Due to the level of expertise required for these techniques, they are often limited to dedicated research laboratories. In a similar vein EM preparations utilising the staining properties of serotonin and the natural density of calcium have also been described, but their use is limited due to the complexity of the preparations (Clauser and Cramer-Borde 2009).

Although TEM is not commonly used in clinical laboratories, with only 7 of 202 ISTH surveyed haematology labs having access to an electron microscope (Gresele et al. 2014), it is a powerful technique which can be used as an adjunct to functional assays in the evaluation of platelet secretion disorders, particularly in the diagnosis of δ-granule-related storage pool disorders.

Immunofluorescence

Fluorophore-conjugated antibodies can be used to identify and visualise the locations of specific proteins in fixed platelet samples. This technique can be extended to platelet secretion by targetting specific cargoes such as vWf, fibrinogen and P-selectin for α-granules, with 5-HT being used as a marker for δ-granules (Fig. 2) (Kamykowski et al. 2011; Sehgal and Storrie 2007; Zufferey et al. 2014). Typically washed platelets are fixed before being cryospun onto coverslip and fixative removed before being permeabilised. Permeabilised platelets are then blocked for a brief period of time before incubation with a primary antibody and subsequently a fluorophore-conjugated secondary antibody. Images can then be generated with the use of a fluorescence microscope—allowing for the evaluation of SPDs. Compared with TEM techniques, it is far easier to label molecules of interest with the use of immunofluorescence, but the overall resolution of the images is greatly reduced. Naturally this technique also relies on the performance of the chosen antibody and may therefore require several optimisation steps. Due to the difficulties associated with this form of imaging, it is often only performed in research laboratories.

Concluding Remarks

Platelet secretion is a major positive feedback mechanism driving haemostasis and thrombosis and is therefore routinely assessed in the evaluation of any suspected platelet disorder (Harrison et al. 2011; Israels 2015). Although there is clearly significant value in assessing platelet secretion, in the management of a range of pathological conditions, and although there is a range of different approaches to assess secretion, there are difficulties associated with many of the methods used. Platelet secretion methods have remained principally the same since their first description some 40 years ago: platelets, either in whole blood, plasma or washed, are activated with agonists, and the release of contents into the medium is quantified—yet, little technological advance has been made in the routinely employed secretion test. While these methods may still be sufficient for the purposes of storage pool disease diagnosis, if our understanding of 'non-canonical' roles of platelets is to improve, some developments are needed.

Clinically, lack of standardisation and poor adoption levels are still an issue with platelet secretion assays—in the recent survey by the ISTH, fewer than half of the 202 surveyed haematology laboratories measured platelet nucleotide secretion in the process of diagnosis of potential inherited platelet function disorders, and only a third used α-granule release methods (Gresele et al. 2014). Guidelines for the diagnosis of inherited platelet disorders recommend granule secretion testing (Gresele and Subcommittee on Platelet 2015), yet no technological recommendations were made.

With the advent of high-resolution microscopy and high-throughput analytical methods such as mass spectrometry, there are the beginnings of progress in the field. The decreasing price of commercially available tests with applications to platelets will also facilitate progress in the field; however, for the time being guidelines on the appropriate use of existing tests are required for wider adoption.

Beyond improvements in the affordability and performance of instrumentation, novel methods to assess platelet secretion are still being investigated. The use of thrombin and prothrombinase generation assays to measure secreted platelet factor V have shown promise in quantifying α-granule secretion (Castoldi and Rosing 2011) and could prove useful in both clinical and research environments with the appropriate standardisation (Berntorp and Salvagno 2008; Dargaud et al. 2012). It has also been suggested that δ-granule secretion could be quantified with the use of the fluorescent dye 4′,6-diamidino-2-phenylindol (DAPI), which binds to secreted polyphosphates and can then be analysed by PAGE (Smith and Morrissey 2007). Whether

these methods are adopted in the clinic to measure platelet secretion will depend on further investigation and optimisation by research laboratories.

Take Home Messages

- As well as being a major contributor to haemostasis and thrombosis, platelet secretion is believed to play a role in numerous other physiological and pathological states including blood vessel formation, cancer, autoimmune diseases and inflammatory conditions.
- Evaluation of platelet secretion can be performed in multiple ways, allowing for quantification of distinct types of platelet secretion—as well as the relative contents of secretory granules.
- Recent advances in technology have made techniques such as flow cytometry and ELISA more readily available, allowing for rapid and reliable detection of platelet secretion from small sample volumes.
- Further understanding of platelet secretion and its contribution to disease states will allow for more sophisticated biomarker development and implementation in a variety of clinical settings.

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Platelet Activation Under Conditions of Flow

Philip G. de Groot and Rolf T. Urbanus

Abstract

Platelets circulate at high velocity through the vasculature. Due to their small size, they are pushed towards the vascular wall. This allows them to rapidly respond to injuries through the formation of a platelet plug and seal off any defect they encounter. Platelets are continuously exposed to shear forces and are well adapted to changes in rheology. They can withstand huge shear stress and are equipped with receptors that allow them to interact with a variety of extracellular matrix proteins. Most of the knowledge we have on platelet function under conditions of flow was obtained with flow chambers. In recent years, flow chambers have evolved to become a versatile platform for studying haemostasis and thrombosis. This chapter describes several extracellular matrix proteins to which platelets can adhere and the receptors they interact with. Furthermore, it provides an overview of the current state of the art with respect to flow chambers.

Introduction

Platelets are small anucleate cell fragments produced by megakaryocytes in the bone marrow. They contribute to the maintenance of the normal circulation by taking care of the integrity of the vessel wall. They circulate in the blood in a resting state until they are needed and can react immediately when necessary. They continuously monitor the vessel wall for injuries. Their surfaces express specific receptors to support this search. When an injury is detected via a receptor, platelets immediately adhere to the exposed subendothelial structures. Here they rapidly activate and aggregate to form a platelet plug that covers the injury. This plug can stop the bleeding temporarily. To ensure complete healing, a fibrin network should stabilize the clot (Ruggeri 2009).

Platelet adhesion is the first step in the haemostatic response, and knowledge of the mechanisms of platelet adhesion can be an important lead for the development of selective antithrombotic drugs. The adhesive proteins responsible for optimal platelet adhesion to injured vessel walls are different from adhesive proteins involved in the adhesion of platelets to other cells, because platelet adhesion has several unique features. Platelet adhesion takes place in flowing blood, which means that the adhesive process must take place rapidly and that the interaction must be firm enough to resist shear forces exerted on the adherent platelet by the flowing blood. This had led to the evolution of adhesive receptors and ligands unique for platelet adhesion. Flow-based assays are indispensable for understanding the dynamics of platelet function.

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Rheology and Platelet Adhesion

Blood is the transport system of the body and flows through the vasculature. Blood flow has a major impact on platelet adhesion. There are two ways in which blood flow influences platelet function: through convection and shear forces (Goldsmith and Turitto 1986). For proteins and cells to initiate a reaction, they need to establish contact with each other. In a test tube, proteins and cells move by diffusion, which is a slow process. To increase the probability of collision in this test tube, reaction substances are always stirred to creating bulk movement of the fluid (convection). Because blood flows, there is a constant supply (and removal) of platelets to the vessel wall, causing a greatly increased number of collisions as compared with static conditions; the greater the flow rate, the higher the number of collisions. In simple reaction kinetics terminology, this means that in the comparative reaction ($V = k$[conc]) the concentration is greatly increased, thus accelerating the reaction. A clear example of this is that the number of platelets that adhere to a surface strongly increases with higher flow rates of the blood (Remuzzi et al. 1985). Only at very high shear rates will the number of collisions become high enough for the reaction kinetics to determine the rate of adhesion. The presence of erythrocytes is important for the adhesion of platelets as well (Aarts et al. 1988). Red cells occupy about 40 % of the blood volume and assemble in the middle of the blood vessel due to their larger size, pushing the smaller platelets outwards to the vascular wall and increasing the concentration of blood platelets near the vessel wall. This implies that a mild bleeding tendency can arise when the haematocrit is low (anaemia).

Shear forces are the forces exerted on the blood cells when they circulate. Shear forces are proportional to the differences in velocity between adjacent fluid layers as they slide along each other. Under laminar flow conditions, the velocity will be greatest in the centre of the blood vessel and zero at the vascular wall. Hence, shear forces are highest near the vascular wall, where the flowing blood interacts with the stationary vascular wall. As this is exactly the site where a thrombus develops, an adhering platelet needs to compensate for these shear forces with a high affinity for vascular wall proteins. To deal with the high shear forces, evolution has provided us with von Willebrand factor (VWF) (Lenting et al. 2015). At low shear stress, blood platelets can adhere to many proteins in the vessel wall. However, when the flow rate increases to arterial blood flow velocities, the presence of VWF is essential for effective primary haemostasis. The mechanism of action of VWF is adapted to its specific function at higher shear forces. VWF is a very long molecule that is irregularly folded in on itself in circulation. In this coiled conformation, the platelet binding sites are not exposed and no interaction with platelets can take place. When VWF binds to the vessel wall, those portions of the molecule that extend outward from the vessel wall are exposed to high shear forces exerted by the blood. This induces the uncoiling of the molecule, leading to the exposure of binding sites for platelets (Siedlecki et al. 1996).

General Mechanism of Platelet Adhesion

The vascular wall is lined with a continuous layer of endothelial cells. Platelets circulate close to the vessel wall without displaying any stable interaction with the endothelial cell lining, because endothelial cells do not express any adhesive proteins that can support platelet adhesion under the shear forces exposed by the flowing blood. Moreover, endothelial cells release prostacyclin and nitric oxide, two low molecular weight components with strong anti-adhesive properties (van Hinsbergh 2012). When the endothelial cell layer is damaged, platelets immediately interact with the exposed subendothelial structures (Ruggeri and Mendolicchio 2015). Stable platelet adhesion requires the interaction between several platelet receptors and matrix proteins. The matrix proteins involved determine the degree of platelet activation and aggregate formation. Which matrix proteins and accessory receptors are involved in the adhesive process depends on the local environment. At higher shear rates, the first interaction of a platelet with the vessel wall will be mediated by VWF (Fig. 1). VWF is present in the subendothelial tissue, in plasma and in the alpha-granules of the platelets and Weibel-Palade bodies of endothelial cells (Savage et al. 2002). After injury, collagens will be exposed to the circulation and the VWF in plasma will immediately bind to these collagens. The initial contact will take place via the GPIb-V-IX receptor complex present on the platelets. This interaction initiates tethering of the circulating platelet to the vessel wall. Driven by shear forces, platelets will roll over VWF in the direction of flow and slow down (Weiss 1995). This enables their interaction with other matrix proteins present in the subendothelium, resulting in firm attachment on the vessel wall. Platelet express many receptors specific for different matrix proteins, the collagen receptors GPVI and $\alpha_2\beta_1$, the fibronectin receptors $\alpha_{IIb}\beta_3$ (historically known as GPIIb:IIIa) and $\alpha_5\beta_1$, the laminin receptor $\alpha_6\beta_1$ and the fibrinogen receptor $\alpha_{IIb}\beta_3$. After adhesion is achieved, the platelet will spread on the surface (Weiss 1995). Spreading is an essential part of the adhesion process, as the multiple interactions of the platelet with the subendothelium enables the platelet to withstand the shear forces exposed by the flowing blood. Each spread platelet provides a new surface for a next platelet to adhere and is the basis of a platelet aggregate. There is a certain similarity between adhesion and aggregation. Both for adhesion and aggregation

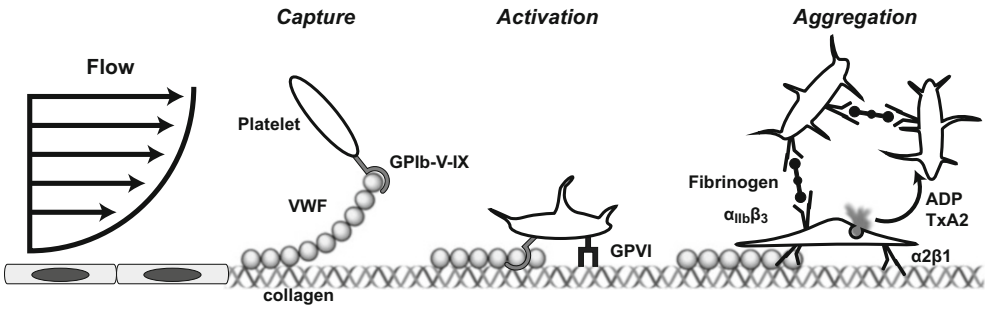

Fig. 1 Platelet adhesion to the injured vascular wall. Platelets are captured from the circulation by collagen-bound VWF through an interaction with the GPIb-V-IX receptor complex. Platelets will tether and adhere to the vascular wall, after which they become activated through the interaction of GPVI with the exposed collagen. The activated platelets will spread on the surface and form tight interactions with VWF through the integrin $\alpha_{IIb}\beta_3$ and with collagen through the integrin $\alpha_2\beta_1$. In addition, they release several soluble agonists, such as ADP and thromboxane A2 (TxA2), which will activate other platelets. This will result in aggregation of platelets

circulating platelets must attach to an adhesive protein, while shear forces are applied on the adhering platelets by the flowing blood. Both VWF and fibrinogen are major players in both processes.

Perfusion Chambers for the Study of Platelet Adhesion

Since the 1970s, major advances have been made in the development of flow chambers for the study of haemostasis. By pumping blood through a chamber with well-defined geometries at a certain infusion rate with a syringe pump, wall shear rates can be accurately controlled. The first flow chambers consisted of a rod on which an inverted dissected blood vessel was placed, which was then placed inside a cylinder through which blood was perfused (Baumgartner and Haudenschild 1972). By varying the distance between the rod and the outer cylinder, shear rates could be controlled. Although this allowed the evaluation of platelet-vessel-wall interactions, the analysis of platelet adhesion and fibrin formation was time consuming and labour intensive. These annular flow chambers were rapidly replaced with parallel platelet flow chambers that consist of two rectangular plates between which the perfusion channel is located (Sakariassen et al. 1983). The wall shear stress (τ) (in Pa) in these chambers depends on the height (h, in m) and width (w, in m) of the chamber, the volumetric flow rate (Q, in m^3/s) and the viscosity of the fluid (μ, in Pa·s) and can be calculated with the following equation (Slack and Turitto 1994):

$$\tau = \frac{6Q\mu}{h^2 w}$$

Flow in these chambers is laminar, as the conditions used to study arterial or venous shear rates are typically associated with a low Reynolds number (Re). Reynolds numbers in rectangular flow chambers can be calculated according to the following equation:

$$\mathrm{Re} = \frac{D_h v \rho}{\mu},$$

in which

$$v = \frac{Q}{A}$$

and D_h is the hydraulic diameter (m), v is the average velocity of the fluid (m/s), A is the cross-sectional area of the flow channel (m) and ρ is the density of the fluid (kg/m^3). For rectangular tubes with a high aspect ratio, the hydraulic diameter equals 2 times the height of the chamber (Fox et al. 2004), as

$$D_h = \frac{2h}{1 + \frac{h}{w}}$$

Flow is considered laminar when the $\mathrm{Re} < 2300$.

One of the plates in a parallel plate perfusion chamber typically consists of a polycarbonate slab, whereas the other plate is a glass or plastic coverslip, which can be coated with a variety of adhesive surfaces. The flow channel can be either cut into the polycarbonate slab or made by placing a silicon gasket in between the two plates. A major advantage of these chambers is that they allow detailed microscopic analysis of thrombus formation. Whereas the first models of the parallel plate perfusion chamber required large amounts of blood to achieve the desired wall shear rate, current designs allow analysis of platelet function with only several hundred microlitres of blood.

With the development of microfluidic devices, the volumes of blood needed to study platelet function have

decreased even further. Soft lithography has allowed researchers to design flow chambers with features down to a few μm, resulting in flow chambers that require microlitres rather than millilitres. In general, soft lithography is based on the fabrication of a positive relief master, on which a replica of polydimethylsiloxane (PDMS) is subsequently moulded (McDonald et al. 2000) (Fig. 2). Researchers can simply design any flow chamber with the dimensions they desire, which is then printed on a transparency at high resolution. This transparency is used as a photomask to create a master on a silicon wafer that has been spin coated with a photocurable epoxy such as SU-8 to the desired height of the flow channel. In this procedure, the photomask is placed in close contact with the wafer, after which the wafer is exposed to UV light. This will induce cross-linking and polymerization of the epoxy substrate. Unpolymerized epoxy is then washed away with a developing solution, after which the master mould is ready for replica moulding with PDMS. Hereto, a volume of PDMS is added to the master mould and cured at 60 °C. The resulting PDMS flow chambers can then be attached to a variety of surfaces, such as glass or the fluoropolymer ACLAR.

The possibilities of PDMS flow chambers are many, as it is relatively easy to create chambers with a stenosis (Nesbitt et al. 2009) or posts that divide the flowpath (Herbig and Diamond 2015). Due to their small size and the excellent optical properties of PDMS, these chambers are highly suitable for analysis of platelet adhesion in real time with differential interference contrast or fluorescence microscopy. Soft lithography has also been used to make scaffolds for 3D cultures of endothelial cells in a hydrogel, leading to the formation of engineered microvasculature with a continuous endothelial lining (Morgan et al. 2013; Zheng et al. 2012). These microvessels can be used to study angiogenesis and the interaction of platelets with the vessel wall.

Adhesive Substrates

Von Willebrand Factor

The gene for VWF is located on the short arm of chromosome 12. Transcription of the VWF gene results in a pre-pro-VWF precursor protein of 2813 amino acids (Ginsburg et al. 1985). The precursor protein is composed of a signal peptide of 22 amino acids, a propeptide of 742 amino acids and a mature protein of 2050 amino acids. The mature protein contains four structurally different domain types, arranged in the sequence D′-D3-A1-A2-A3-D4-C1-C2-C-C4-C5-C6-CK (Zhou et al. 2012). VWF is a multimeric protein and this multimerization is essential for its function. Two VWF subunits are dimerized via a C-terminal disulphide bridge in the endoplasmic reticulum. A second disulphide bridge is subsequently formed in the trans-Golgi network via the N-terminal D′-D3 domains, which requires the release of the propeptides (Vischer and Wagner 1994). VWF is synthesized by endothelial cells and megakaryocytes. In both cases, VWF is targeted to storage organelles: Weibel-Palade bodies in endothelial cells and α-granules in platelets (Sporn et al. 1985; Wagner et al. 1982). It can be released

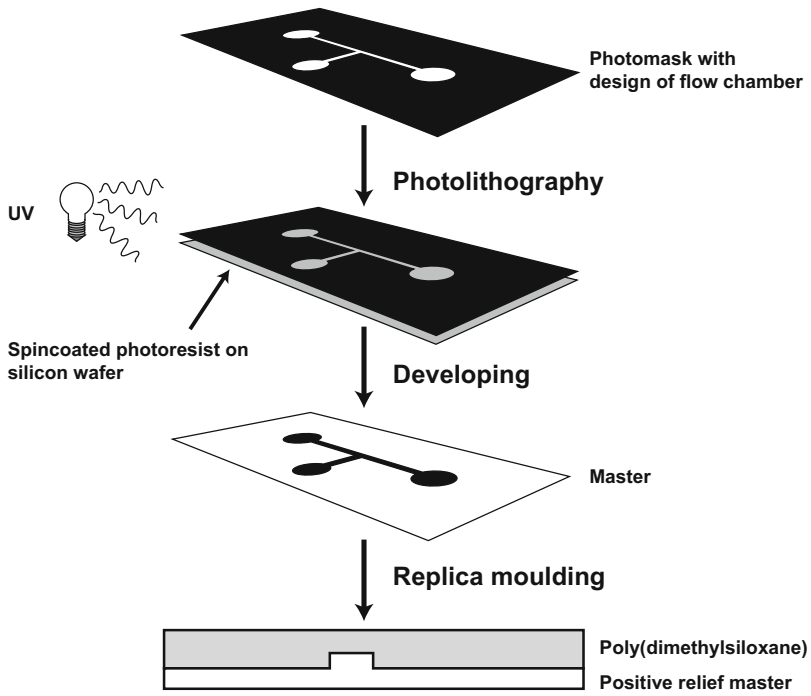

Fig. 2 Soft lithography for the design of microfluidic flow chambers. A design of a microfluidic device is printed on a transparency at high resolution. This is placed on a silicon wafer that has been spin coated with a photocurable epoxy to the desired height of the microfluidic device. The epoxy polymerizes after exposure to UV light, after which the unpolymerized epoxy is washed away with a developer solution. The resulting positive relief master can be used for replica moulding of hundreds of poly(dimethylsiloxane) microfluidic devices

from both storage sites upon activation of these cells. After release, VWF can self-associate with VWF from the circulation, creating extremely large fibres (Savage et al. 2002). Spontaneous self-association of plasma VWF has been described to occur in regions of flow deceleration under high shear conditions (Nesbitt et al. 2009) and can be mimicked in flow chambers with a stenosis or posts that divide the flowpath (Herbig and Diamond 2015). These fibres are an ideal runway for the circulating platelets. Self-association may involve thiol-disulphide exchange (Choi et al. 2007). Apart from activated release, endothelial cells also secrete VWF constitutively. This process is thought to be the predominant source of VWF in plasma. Experiments in flow chambers with cultured human endothelial cells show that stimulation of these cells with a secretagogue such as histamine induces the release of ultralarge VWF (Dong et al. 2002). These VWF fibres can span several millimetres in the absence of the protease ADAMTS13 (Zheng et al. 2015), a condition encountered in patients with thrombotic thrombocytopenic purpura (TTP). This endothelium-suspended ultralarge VWF provides an adhesive surface for platelets that can be visualized as "beads on a string" (Fig. 3). However, secreted VWF will be rapidly cleaved in the presence of ADAMTS13, which prevents platelet adhesion (Dong et al. 2002).

VWF has three major functions. It is essential for the adhesion of platelets to injured vessel walls, it is involved in platelet-platelet interaction at higher shear rates and it is the carrier protein in plasma for factor VIII. Without VWF, factor VIII is rapidly cleared from the circulation (Lenting et al. 2010). VWF circulates in plasma with an average plasma concentration of 10 μg/mL and the average concentration in platelets is 280 ng/10^9 platelets (Rodeghiero et al. 1992). The normal range of plasma VWF is broad and is strongly influenced by the ABO blood group, with 25 % lower levels in blood group O and higher levels in blood group AB (Sarode et al. 2000). Interestingly, platelet VWF is independent of blood group (Matsui et al. 1999). Low VWF levels will result in a bleeding tendency while high levels of VWF are correlated with a thrombotic tendency.

After a vascular injury, circulating VWF immediately binds to the exposed collagens present in the subendothelium via the A3-domain (Romijn et al. 2003). Binding of VWF results in the exposure of a cryptic epitope in the A1-domain of VWF, which will be recognized by GPIbα (Auton et al. 2010), one of the subunits of the GPIb-V-IX complex on the platelet membrane. This interaction will result in rolling of platelets over VWF in the direction of flow, allowing the platelet to interact with other matrix protein to reach stable interaction. During this rolling process, the platelet will interact with VWF through other receptors as well. One of these receptors is the $\alpha_{IIb}\beta_3$ integrin, which can bind an RGD sequence in the C4-domain of VWF (Berliner et al. 1988). The importance of the interaction of VWF with the GPIb-V-IX complex is illustrated in patients with Bernard-Soulier syndrome who fail to produce a functional GPIb-V-IX complex (Ware et al. 1993) and have a moderate to severe bleeding tendency.

The crystal structure of the complex of the VWF A1-domain and the N-terminal fragment of GPIbα has been solved (Huizinga et al. 2002). The crystal structure of the complex shows that the globular A1-domain interacts with the concave face of GPIbα with two distinct areas of tight interactions, one at the top and one at the bottom of the A1-domain. One would expect that the lifetime of the bond between GPIbα and VWF would diminish with force. However, there are indications that the bond between GPIbα and VWF increases with increased shear stress. This would suggest that the interaction between GPIbα and VWF behaves as a catch bond (Yago et al. 2008), in which interactions become stronger when force is applied. However, detailed molecular analyses of the interaction between VWF-A1 and GPIbα indicate that the bond between the two molecules switches between two distinct states with slip-bond characteristics, a low-affinity state and a high-affinity state (Kim et al. 2010). Application of force to the bond between VWF and GPIbα causes the transition of the low-affinity state to the high-affinity state, probably due to a conformational change in VWF.

Binding of GPIb-V-IX complex to VWF induces platelet activation, converting $\alpha_{IIb}\beta_3$ into a high-affinity receptor for different ligands (Andrews and Berndt 2004). This is an essential step in platelet spreading and stable adhesion. How GPIb-V-IX contributes to platelet activation and thrombus formation is controversial and seems to be dependent on the shear stresses exposed.

Patients with low or absent VWF suffer from a bleeding tendency known as von Willebrand disease (VWD)

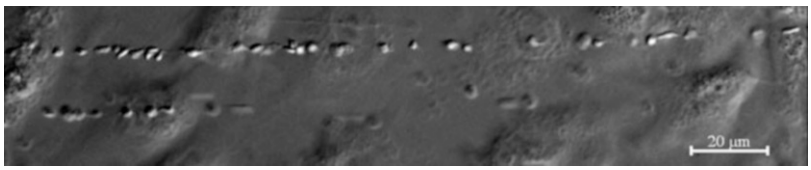

Fig. 3 Platelets adhere to VWF strings attached to the endothelium. Endothelial cells were grown to confluence and stimulated with histamine. Washed platelets were perfused over the endothelial cells at a shear rate of 100 s^{-1}

(Ng et al. 2015). VWD is classified into three major types. VWD type I is characterized by a partial deficiency of VWF and is the most common type. Nowadays plasma levels of VWF between 20 % and 50 % are considered a risk factor for bleeding, while VWD type I is diagnosed when VWF levels are below 20 %. Type III is a very rare condition in which VWF levels are below 1 %. In contrast to types I and III, which are quantitative deficiencies, type II VWD is characterized by qualitative defects. Type II is divided into four subtypes. Type 2A is characterized by deficient large multimers and consequently a reduced function. Type 2M is the result of a mutation in the A1-domain of VWF resulting in a loss of interaction with GPIb-V-IX complex. Type 2M has a normal multimer pattern. Type 2B is characterized by an increased affinity of VWF for GPIb-V-IX complex. As the consequence of the mutation, the cryptic epitope in the A1-domain of VWF is already expressed in the circulating plasma VWF. As a result patients also suffered from thrombocytopenia and the highest multimers of VWF are absent. Type 2N mutations result in deficient factor VIII binding and haemophilia A-like symptoms.

Collagens

Collagens are distinguishable from other extracellular matrix proteins by their triple-helical conformation. All fibrillar collagens have a very similar tertiary protein structure and are composed of three peptide chains (α-chains). The amino acid sequences of these α-chains show two unique features. There is a glycine residue at every third position, generating a repeating $(Gly\text{-}X\text{-}Y)_n$ pattern, and there is a high proportion of proline and hydroxyproline (Hyp) on the X and Y position, respectively. These features are essential to establish a stable triple helix (Farndale et al. 2003). The triple helix conformation always consists of three parallel polypeptide chains that are staggered by one amino acid residue. The glycine residues are buried in the backbone of the helix while the X and Y are exposed on the outside. The amino acids on the X and Y position determine the specificity of collagens for certain interactions. Platelet GPVI recognizes a repeat of the Gly-Pro-Hyp motif (Morton et al. 1995), and $\alpha_2\beta_1$ recognizes a Gly-Phe-Hyp-Gly-Glu-Arg motif (Knight et al. 1998). VWF specifically recognizes an Arg-Gly-Gln-Hyp-Val-Met-Gly-Phe sequence, a highly evolutionary conserved sequence (Lisman et al. 2006). Interestingly, this complex sequence overlaps with the binding site for a number of other proteins, such as SPARC (osteonectin) and discoidin domain receptors 1 and 2 (Hohenester et al. 2008). This sequence is not present in collagen type I, and analysis of a crystal structure of the peptide with the A3-domain of VWF showed that VWF recognizes amino acids from different collagen I polypeptide chains present in the same helix structure (Brondijk et al. 2012).

Collagens are the most abundant proteins in mammals. The collagen family comprises 28 members that contain at least one triple-helical domain (Mienaltowski and Birk 2014). At least seven different collagens have been identified in the vessel wall, of which collagen types I, III, IV and VI are able to support platelet adhesion. Collagens are insoluble proteins, and the methods used to solubilize them in order to test their reactivity in vitro could influence their structural integrity. The fibrillary structures of collagens in vivo display a different banded pattern compared to the in vitro formed fibrils (Farndale et al. 2004). Also, interactions of collagens with other matrix proteins might influence their reactivity towards platelets. Matrix metalloproteinases will degrade the fibres which will result in disappearance or exposure of reactive sites (Guglielmini et al. 2015). The affinity of collagen receptors for collagens strongly depends on the conformation of the collagen fibrils. The in vitro experiments on platelet interaction with different types of collagen might underestimate the role of collagens in vivo. Experiments with genetically manipulated collagens in animal models should help us to understand the exact role of collagen in platelet adhesion.

Although several collagen receptors on platelets have been proposed (Nuyttens et al. 2011), GPVI and $\alpha_2\beta_1$ are the only collagen receptors that have been shown to be relevant because deficiencies of these receptors result in mild bleeding disorders. Other proposed receptors such as GPIV (CD36) seem unlikely, because 5 % of the Japanese population is deficient for this receptor without consequences for their platelet function (Masuda et al. 2015). Platelet adhesion to collagen is strongly affected by the presence of Mg^{2+} and Ca^{2+} (van Zanten et al. 1996). Platelet adhesion to collagen is stimulated by the presence of Mg^{2+} whereas Ca^{2+} has an inhibitory effect.

Experiments with triple-helical collagen peptides have indicated shear-dependent differences in the requirements for collagen receptors during platelet adhesion. The VWF binding site enhances thrombus formation at all shear rates, but is only required at high shear rates ($>3000\ s^{-1}$). Whereas the presence of both the integrin $\alpha_2\beta_1$ and GPVI binding sites is necessary for platelet adhesion at a low shear rate, the presence of either binding site in combination with the VWF binding site is sufficient for platelet adhesion at a shear rate of $1000\ s^{-1}$ (Pugh et al. 2010).

Fibrinogen

Fibrinogen is not a structural component of the vessel wall, as it is not synthesized by vascular cells. Nevertheless, it is

present in the vessel wall as the result of adsorption from plasma. Platelet adhesion to fibrinogen has received much attention, because fibrinogen is the dominant plasma protein that adsorbs to biomaterials such as stents and grafts. The adsorbed fibrinogen provides an ideal surface for platelet adhesion (Hantgan et al. 1990) (Fig. 4). Moreover, the adhesion to a fibrin network is a key event in the haemostatic response (Miszta et al. 2014). The integrin $\alpha_{IIb}\beta_3$ is the major receptor for both fibrinogen and fibrin at all shear rates. Fibrinogen has three potential epitopes that are able to interact with $\alpha_{IIb}\beta_3$. There are two RGD sequences in the α-chain of fibrinogen and an AGDV sequence at position 407–411 of the γ-chain. Different studies have shown that the RGD sequences are not important for platelet adhesion, while the sequence in the γ-chain is essential (Hantgan et al. 1995; Zaidi et al. 1996). The binding of soluble fibrinogen to platelets only takes place after stimulation of the platelets. Stimulation of platelets opens the $\alpha_{IIb}\beta_3$ integrin. However, fibrinogen that is adsorbed to a surface supports the adhesion of non-stimulated platelets via the $\alpha_{IIb}\beta_3$ integrin (Savage and Ruggeri 1991).

Platelet adhesion to fibrin and fibrinogen at high shear rates not only depends on $\alpha_{IIb}\beta_3$ integrin, but also on a secondary interaction between the GPIb-V-IX complex and VWF (Endenburg et al. 1995). This latter interaction is necessary for the adhered platelet to withstand the shear forces. Recent studies have shown that VWF is incorporated into fibrin in the presence of thrombin (Miszta et al. 2014). Because fibrin is rapidly formed in vessels with low shear rates, the contribution of fibrin to the adhesion process might be more relevant in the venous circulation. A recent study has identified GPVI as a third platelet receptor for polymerized fibrin (Mammadova-Bach et al. 2015). Due to its function in platelet activation, GPVI-fibrin interaction results in amplification of thrombin generation. Earlier studies failed to identify the proposed role of GPVI in the adhesion of platelets, probably because all in vitro studies on platelet adhesion to fibrin were performed in citrated blood. Interestingly, a single nucleotide polymorphism in GPVI was identified as a risk factor for venous thrombosis in a large study on genetic variants associated with venous thrombosis (Bezemer et al. 2008).

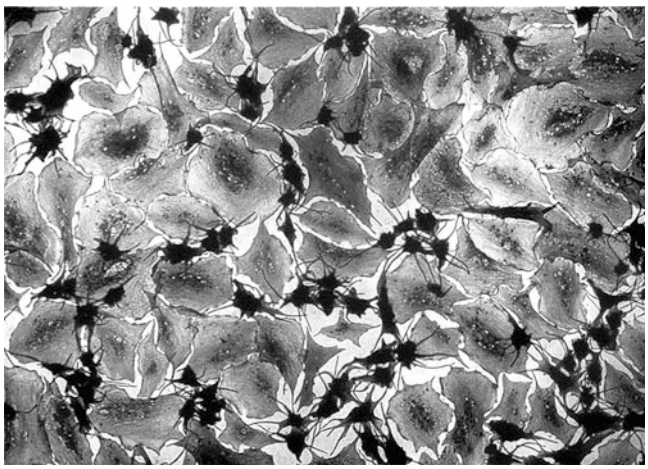

Fig. 4 Platelet spreading on a fibrinogen-coated surface. Human platelets were allowed to adhere to fibrinogen for 30 minutes

Fibronectin

Fibronectin is a ubiquitous glycoprotein composed of two structurally similar but not identical subunits of approximately 220 kDa, linked by a pair of disulphide bonds. The two chains are different due to alternative splicing in the so-called III connecting segment (Huelsz-Prince et al. 2013). Fibronectin is present in two forms: as a soluble plasma form and as a high-molecular-weight aggregate that is present in tissues. Fibronectin is an essential adhesive protein and involved in many fundamental biological processes. Platelets contain two receptors for fibronectin on their surface, $\alpha_5\beta_1$ and $\alpha_{IIb}\beta_3$ (Ginsberg et al. 1993). Mice without $\alpha_{IIb}\beta_3$ are unable to form platelet thrombi under these conditions (Smyth et al. 2001), suggesting that fibronectin can function as an adhesive protein for platelets. Indeed, mice conditionally deficient in fibronectin show delayed thrombus formation (Ni et al. 2003). However, mice triple deficient in VWF, fibrinogen and fibronectin show more platelet adhesion than mice deficient in VWF and fibrinogen, suggesting that fibronectin inhibits the adhesion process (Reheman et al. 2009). Different roles of plasma and tissue fibronectin might complicate the whole issue. In vitro, fibronectin is a rather poor substrate for platelet adhesion (Beumer et al. 1994). Interestingly, a splice variant of fibronectin known as EDA-fibronectin that is normally present in the extracellular matrix but is detected in plasma in certain diseases has been found to increase in vivo and in vitro thrombus formation (Chauhan et al. 2008). There are at least 20 different isoforms of fibronectin in humans (White and Muro 2011). The exact function of fibronectin in platelet-vessel wall interactions might be difficult to elucidate due to the presence of all of these isoforms.

Thrombospondin

The thrombospondin family consists of five members (Halper and Kjaer 2014). Platelets contain thrombospondin-1 in their α-granules. After platelet activation, thrombospondin-1 is secreted, binds to the platelet membrane and mediates adhesion (Wencel-Drake et al. 1985). In vitro, thrombospondin-1 can support platelet adhesion at high shear rates (up to 4000 s^{-1}). The primary receptor involved in the adhesion

to thrombospondin-1 is GPIb-V-IX; however, the adhesion to thrombospondin-1 seems to be independent of VWF (Jurk et al. 2003). A secondary role for GPIV (CD36) has been suggested. The subendothelium contains thrombospondin-2 and its absence in mice has been associated with a haemostatic defect (Kyriakides et al. 1998). However, whether this is a causal relation is not certain and the significance of the thrombospondins for optimal platelet adhesion is not known.

Laminin

Laminin is a ubiquitous basement membrane protein. Two forms of laminin, laminin 8 and laminin 10, are highly expressed in the subendothelium. Platelets contain laminins 8, 10 and 11, which are secreted after activation (Nigatu et al. 2006). In vitro, the laminin molecule shows a cross-shaped structure with a molecular weight of 850 kDa. It is build up of three distinct chains, 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 $\alpha_6\beta_1$ (Hindriks et al. 1992). Adhesion to laminin is strongly dependent on the presence of Mg^{2+} and Ca^{2+}. Platelet adhesion to laminin via $\alpha_6\beta_1$ does not result in activation of platelets. The interaction of GPVI with laminin is reported to mediate platelet activation after adhesion to laminin, resulting in lamellipodia formation (Inoue et al. 2006).

Vitronectin

The contribution of vitronectin to thrombus formation is controversial. Mice deficient in vitronectin show impaired thrombus formation that can be restored by administration of vitronectin (Reheman et al. 2005). Additional experiments show that thrombi formed in the absence of vitronectin are unstable, suggesting that vitronectin is involved in platelet-platelet contacts and not in primary adhesion of platelets to the vessel wall (Wu et al. 2004).

Platelet Interaction with Other Components of the Vessel Wall

In general, all tissue components that are able to interact with platelets can contribute to platelet adhesion and thrombus formation. The extracellular matrix is a complex system in which many proteins and proteoglycans interact with each other. These interactions might also involve binding sites that are thought to be involved in platelet adhesion. Some proteins might modulate platelet interaction with the vessel wall, which will never be observed in experiments with purified proteins. As said, purification of the often insoluble proteins could also influence their adhesive properties. Moreover, some proteins exist in plasma as well as the subendothelium and both forms are not necessarily identical. Experiments with the plasma form of the protein not always reflect the properties of the matrix form. Experiments with purified proteins will teach us about the receptors involved and the subsequent consequences for the activation of the platelet, but whether it reflects the in vivo situation remains to be established.

Regional Differences Within the Growing Thrombus

With the improvement of visualization techniques, our view of the growing thrombus has changed significantly. Traditionally, the growing thrombus was viewed as a mass of activated platelets interspersed with fibrin fibres, wherein several activation pathways work in concert throughout the growing thrombus to create a haemostatic plug. Studies with high-resolution real-time in vivo confocal microscopy have led to a nuanced view of the same process, as there appear to be large regional and temporal differences in platelet activation status and in the availability of soluble platelet agonists within the thrombus. By injection of fluorophore-conjugated antibodies against P-selectin, fibrin or platelet markers such as GPIbα or the α_{IIb} integrin subunit into the blood stream of the mouse, it has become possible to study the temporal and spatial differences in platelet activation upon vascular injury (Dubois et al. 2007; Falati et al. 2002; Hayashi et al. 2008; Stalker et al. 2013). We now know that those platelets that are directly adhered to the site of injury are fully spread, have released the content of their granules and form a tight, densely packed, interconnected mass. Together, they form the dense core of the growing thrombus (Dubois et al. 2007; Hayashi et al. 2008; Stalker et al. 2013). The layers of platelets that are added to this dense core region by the flowing blood are progressively less activated, depending on their distance from the site of injury. The platelets in this boundary layer have not released the content of their granules and make up the shell of the thrombus (Stalker et al. 2013). In vitro models with side-view microfluidic flow chambers show a similar thrombus architecture and are promising tools for further investigation into the dynamics of thrombus formation in human samples (Welsh et al. 2012).

By using fluorescently labelled proteins or dextrans of varying molecular weight, it has become possible to study differences in packing density in the growing thrombus (Stalker et al. 2013). Platelets will contract during the consolidation of the dense core of the thrombus, resulting in even smaller gaps between adjacent platelets through which soluble agonists can diffuse. This results in the formation of

a microenvironment that is protected from the influences of shear stress and blood flow and causes the retention of locally produced agonists (Welsh et al. 2014). Thrombin formation appears to be limited to the core of the growing thrombus and the surrounding endothelium (Ivanciu et al. 2014; Welsh et al. 2012). This makes sense, as the relatively large size of thrombin would prevent its diffusion from the densely packed platelets at the core of the thrombus. The soluble platelet agonists ADP and thromboxane A2 are much smaller and will diffuse readily through the different layers of activated platelets. As only the platelets present in the core of the thrombus express P-selectin, these platelets are the source of ADP (Dubois et al. 2007; Hayashi et al. 2008; Stalker et al. 2013). The ADP that diffuses from this layer causes mild activation of the platelets in the shell region of the thrombus, but does not lead to granule secretion, allowing further growth of the thrombus. The importance of ADP in growth of the shell region is illustrated by the consequences of inhibition of ADP-induced platelet activation with P2Y12 antagonists (Fig. 5). Whereas the densely packed, P-selectin positive core region remains similar in size under these conditions, the size of the shell region is substantially reduced (Stalker et al. 2013). Conversely, thrombi formed in mice with a gain-of-function mutation in $G_{i\alpha}$ that renders platelets more sensitive to stimulation of P2Y12 show a marked increase in shell size (Stalker et al. 2013). These observations explain the relative safety of P2Y12 antagonists over $\alpha_{IIb}\beta_3$ antagonists.

The Interplay Between Coagulation and Platelets

Collagens and thrombin are widely regarded as the most important platelet agonists in vivo. When platelets are captured from the circulation and firmly adhere to the site of injury, they will be rapidly activated through the interaction of GPVI with the exposed subendothelial collagens, which leads to both $\alpha_{IIb}\beta_3$ activation and granule release. At the same time, thrombin formation will be initiated in the extravascular tissue and the damaged endothelium immediately adjacent to the site of injury. Thrombin will cleave fibrinogen, leading to fibrin formation, and will cause additional platelet activation. Studies with Fcγ-knockout mice lacking a functional GPVI collagen receptor emphasize the relatively minor contribution of GPVI in the formation of the platelet plug, as stable thrombi formed in the absence of GPVI (Mangin et al. 2006). Moreover, congenital deficiency of GPVI is usually associated with a mild bleeding phenotype in humans (Kojima et al. 2006; Moroi et al. 1989; Takahashi and Moroi 2001). The importance of thrombin in the formation of a stable thrombus is illustrated by the greatly decreased expression of P-selectin in the region covering the site of injury in mice treated with hirudin (Stalker et al. 2013). As the thrombi formed under these conditions mostly consist of mildly activated platelets, they are likely to embolize and will eventually be sheared away by the flowing blood.

Anticoagulation

Traditionally, platelet adhesion is studied in blood anticoagulated with calcium chelators such as sodium citrate or coagulation inhibitors such as heparin, pentasaccharide, Phe-Pro-Arg-chloromethylketone (PPACK) and the newer direct oral anticoagulants, thereby fully excluding the influence of thrombin and fibrin on the formation of thrombi. The evidence derived from animal models has prompted researchers to further investigate the complexities of the interplay between coagulation and platelets. Ex vivo, this can be achieved when blood is drawn directly from the antecubital vein of the donor through the flow chamber, but these kind of experiments usually require large volumes of blood and are associated with significant contact activation in the tubing (Heyligers et al. 2006; Kirchhofer et al. 1994; Orvim et al. 1997). For this reason, most researchers prefer the use of (minimally) anticoagulated blood. During blood collection, contact activation will rapidly cause clotting. Collecting blood in tubes with corn trypsin inhibitor

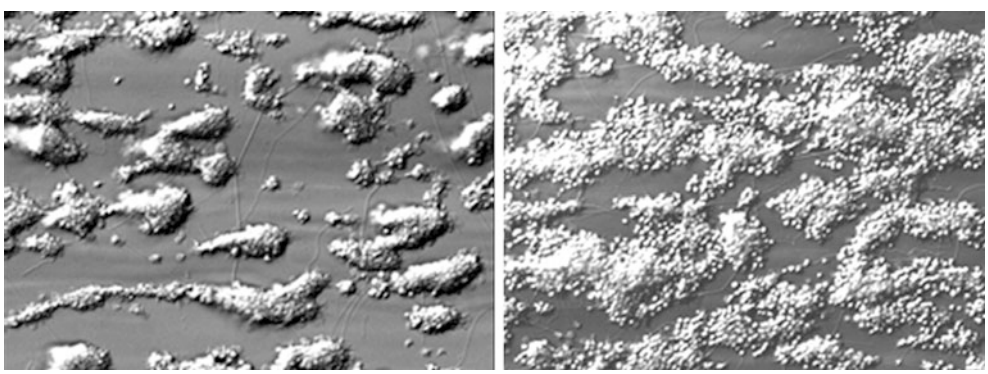

Fig. 5 P2Y12 inhibition greatly reduces thrombus height. Whole blood from a healthy donor (*left*) and a donor receiving clopidogrel treatment (*right*) was perfused over a collagen-coated surface at 1200 s^{-1} for 5 minutes, after which the thrombi were perfused with buffer

(CTI), a specific FXIIa inhibitor, will postpone contact activation, but does not prevent the activation of FXII entirely (Rand et al. 1996). Samples collected in CTI should therefore be processed within minutes of venepuncture. Newer inhibitors of contact activation such as direct FXIIa inhibitors such as infestin-4 (Hagedorn et al. 2010) or blocking anti-FXI antibodies (Cheng et al. 2010) are currently under investigation. An alternative to blocking the contact pathway is the recalcification of citrated whole blood. Trisodium citrate chelates Ca^{2+}, Mg^{2+} and Zn^{2+} ions but leaves metal ion-dependent proteins such as integrin $\alpha_{IIb}\beta_3$ intact. Recalcification with a mixture of Ca^{2+}, Mg^{2+} and Zn^{2+} fully restores the coagulation potential of a blood sample at the expense of dilution.

Coagulation Under Conditions of Flow

Physiologically relevant coagulation reactions start with the exposure of blood to tissue factor (TF). TF has been introduced in flow experiments in several ways. Endothelial cells have been grown to confluence on glass coverslips, stimulated with phorbol 12-myristate 13-acetate (PMA) or TNFα to fully activate the quiescent endothelium and subsequently lysed with NH_4Cl to create subendothelial matrix that contains TF, as well as VWF and fibronectin (Zwaginga et al. 1990). Others have directly adsorbed lipidated TF onto the wall of the flow channel or attached lipidated TF to the immobilized collagen (Welsh et al. 2012). As there is evidence for a surface TF threshold for fibrin formation, it is important to calculate TF surface density (Okorie et al. 2008). By conjugating a fluorescent thrombin sensor to an antibody that targets the platelet surface, thrombin formation can be visualized in real time in the growing thrombus (Welsh et al. 2012). Whether or not the targeting of the thrombin sensor to platelets provides an accurate representation of thrombin formation in vivo remains to be determined, as recent evidence suggests that the prothrombinase complex is primarily present on the endothelium directly adjacent to the vascular injury and not on the platelets present in the thrombus (Ivanciu et al. 2014). The role of the contact system in thrombus formation has received renewed attention since the description of the protective effect of FXII-deficiency on thrombus formation in murine arterial thrombosis models (Renné et al. 2005). Polyphosphates are stored in platelet dense granules and are released after platelet activation (Ruiz et al. 2004). They have been shown to support contact activation (Muller et al. 2009), but have also been shown to enhance coagulation reactions independent of FXII (Morrissey et al. 2012). Most of the data on the role of FXII and polyphosphates in thrombus formation are derived from murine models, and translation of these findings to the human situation is difficult. Flow chambers with TF- and collagen-coated surfaces provide a means to study the role of platelet polyphosphates during thrombus formation under standardized conditions. Data derived from such studies indicate that the effect of polyphosphate on thrombus formation can occur independent from FXII (Zhu et al. 2015).

Conclusions

Platelet adhesion under conditions of flow is a complex process in which many adhesive proteins and receptors participate. The development of new perfusion devices has led to major advances in our understanding of the mechanisms that are responsible for platelet thrombus formation on injured vessel walls in flowing blood. The in vitro results obtained with perfusion chambers and purified matrix proteins in the past are now challenged by observations made with genetically modified mice. We now know that there are major regional differences in platelet interaction with the vessel wall and we begin to understand the roles the different adhesive proteins and receptors play locally. But there is still a lot to do. There is an ongoing debate on how the different matrix proteins are involved in the activation of the adhered platelets. Until now, we have focused on the prothrombotic roles of adhesive proteins without thinking on the possibility that some proteins might modulate the prothrombotic properties of other adhesive proteins (Agbanyo et al. 1993). Our knowledge on the interplay between platelet function and coagulation is still limited. The observation that patients with Glanzmann's disease can be efficiently treated with factor VIIa points to an intense interaction (Poon 2007). The role of fibrinolysis is underestimated as well. Finally, thrombus formation predominantly takes place on atherosclerotic vessels and our knowledge on the changes that have taken place in the composition of the subendothelial structures in an atherosclerotic vessel are limited. The newly developed flow chambers in which a stenosis or a bifurcation has been introduced will teach us about the specific influences a sudden change in shear forces will have on thrombus formation.

Take Home Messages

- Platelets can interact with a variety of extracellular matrix components.
- Soft lithography in combination with positive relief moulding provides a versatile means for the manufacturing of microfluidic devices from poly (dimethylsiloxane) (PDMS).

(continued)

- Advancements in visualization techniques and real-time video microscopy have led to a better understanding of thrombus dynamics.
- With the development of disposable microfluidic devices, it has become possible to study the interplay between coagulation and platelets in vitro.

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In Vivo Imaging of Thrombosis and Hemostasis

Anil K. Chauhan

Abstract

Hemostasis and thrombosis are complex processes, which require blood cellular components as well as plasma components. In vivo imaging is a powerful tool that is beginning to provide quantitative and dynamic insights into the field of thrombosis and hemostasis. This chapter provides an overview, how in vivo imaging provides an opportunity to complement and confirm data generated from in vitro studies in the physiological context.

Introduction

In the last two decades, in vivo imaging has significantly contributed to advancing our understanding of the field of hemostasis and thrombosis. Although in vitro experiments are instrumental in asking fundamental questions about mechanisms at the cellular or molecular levels, they fail to take into account the complex architecture and physiology of multicellular tissues. For this reason in vivo imaging was developed to allow researchers to test concepts generated in vitro and provide new insights in the physiological context. The aim of this chapter is to highlight the power of in vivo imaging to address questions in the context of thrombus formation that cannot otherwise be answered, due to the limitations of in vitro studies. I will provide an overview by discussing instrumentation and other components required for in vivo imaging, types of vessels studied in models of experimental thrombosis, methods of inducing vascular injury, effects of mechanical ventilation in experimental thrombosis, and methods of visualizing and quantifying data.

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Equipment for In Vivo Imaging

To image platelet adhesion and thrombus formation in real time, one requires a wide-field microscope (inverted or upright), appropriate objectives, a bright or fluorescent light source, a high-speed camera, software, and a computer to acquire images. Microscopes can be equipped with a motorized XY stage that allows precise computer-controlled lateral movement between XY positions and a Z control to allow rapid changes in the focal plane. The in vivo imaging systems in our laboratory are developed utilizing a Nikon F1 fixed stage (upright microscope) and a Nikon TI-U inverted microscope (Prakash et al. 2015a, b; Dhanesha et al. 2015). The upright microscope has CF1 Fluor 10×, 20× (numerical aperture (NA), 0.50), 40× (NA, 0.80), and 60× (NA, 90) water-immersion objectives. The inverted microscope has CFI Super Fluor (10×, 20×, and 40×) objectives. The microscopes have motorized Z control and are equipped with a Lambda DG-4 high-speed wavelength switcher based on dual scanning galvanometers from a 175-W xenon light source with ET-Sedat Quad filters (402 ± 8 nm, 490 ± 10 nm, and 555 ± 12 and 645 ± 15 nm) matched to individual filter sets for DAPI, FITC, Cy3, and Cy5 (Chroma, Brattleboro, Vermont) in the body of the microscope. These components allow for simultaneous collection of images in two or three fluorescence channels. Images are collected through a high-speed camera (EM) that captures up to 30 frames/s or up to 60 frames/s using 2 × 2 binning. The microscope imaging software from Nikon

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(NIS element) is used for offline data analysis. The software can merge acquired images from each channel automatically, and the fluorescence of each channel can be independently adjusted using a renormalization tool of the software.

Some laboratories use high-speed confocal and wide-field microscopes for in vivo imaging of thrombus in the cremaster muscle vasculature, which was first developed by the Furie laboratory group (Falati et al. 2002; Welsh et al. 2014). The imaging system is developed utilizing an Olympus AX70 microscope equipped with a trinocular head, long-distance condenser, and 40× (NA, 0.80) and 60× (NA, 90) water-immersion objectives. For confocal fluorescence microscopy, a Yokogawa CSU-10 confocal scanner (Perkinelmer, Gaithersburg, Maryland) is used. The imaging system is equipped with an argon-krypton 3-line laser for excitation at 488, 568, and 647 nm and a Sutter Lambda L-10 filter wheel (Novato, California) on the excitation source. Wide-field in vivo microscopy is carried out using a Sutter Lambda DG-4 high-speed wavelength changer with a 175-W xenon light source equipped with excitation filters (360, 480, and 590 nm) matched to a triple-band filter for DAPI, FITC, and Cy5 (Chroma, Brattleboro, Vermont) in the body of the microscope. To provide a bright-field channel while collecting image data in fluorescence channels, a Uniblitz (Vincent Associates, Rochester, New York) shutter is used for transmitted light. For both confocal and wide-field imaging, light is amplified using a videoscope image intensifier. A Cooke SensiCam CCD camera (640 × 480 format) is used to capture up to 60 frames/s using 2 × 2 binning. A Dell workstation equipped with SlideBook software (Intelligent Imaging Innovations, Denver, Colorado) is used for data capture, component control, and image analysis.

Types of Blood Vessels Used for In Vivo Imaging

The mouse is a common choice for in vivo imaging because 99 % of human genes have orthologs in mouse, and they are easy to manipulate genetically, thus making them an ideal "model organism" to study function of human genes in health and disease. With their small size, short gestation period, and ease of breeding, maintenance of mice is simple, feasible, and inexpensive. Although there are other species that are more closely related to humans (such as dogs, pigs, and nonhuman primates), it is more difficult to perform in vivo imaging in such species because of the larger size of the vessels (which impedes light transmission), long gestation period, and lack of efficient methods for genetic manipulation. In order to perform in vivo imaging of thrombus formation in small or large vessels, the mouse must remain anesthetized for 45 min to 2 h depending on the experiment. The most commonly used anesthesia is a mixture of ketamine (60–100 mg/kg) and xylazine (10–50 mg/kg), which is injected intraperitoneally. Alternative anesthetics included pentobarbital (50 mg/kg, IP), midazolam (0.3–0.5 mg/kg, SC), or continuous inhalation of isoflurane (1–3 %) mixed with medical air. Some of the most common vessels that are used by researchers for imaging thrombosis in vivo are described below:

1. *Carotid artery* (Prakash et al. 2015b; Dhanesha et al. 2015): Mice (age 8–10 weeks) are anesthetized, and an incision is made to carefully expose the right or left common carotid artery using blunt dissection. The artery is carefully dissected and made free of any surrounding tissue or fat before the animal is placed on the upright microscope platform. Care should be taken to keep the carotid artery moist by superfusion with warm phosphate-buffered saline (37 °C). One can attach a Doppler flow probe to measure flow rate, which ranges from 1300 to 1800 s^{-1}. The carotid artery is visualized using a water-immersion lens. Following injury, time-lapse images are recorded for later quantification. Advantages of the carotid artery include: (1) dissection of the muscle does not traumatize carotid vessel, (2) mechanical injury can be induced, (3) a Doppler flow probe can be used to calculate flow rate, and (4) the method has clinical relevance to thromboembolic stroke. Disadvantages are that it is not possible to record bright-field images because of the tissue underlying the vessel, and only fluorescent images can be taken.
2. *Mesenteric vessels* (Denis et al. 1998; Chauhan et al. 2006, 2008; Ni et al. 2000, 2003): Younger mice (3–4 weeks) are used because older mice have more fat around the mesenteric vessels, which makes it difficult to image them. An incision is made in the anesthetized mouse through the abdominal wall to expose the mesentery. Arterioles of diameter approximately 100–150 μm with shear rates of ~1300–1800 s^{-1} and venules of diameter 150–400 μm with shear rates of 100–300 s^{-1} are placed on a Plexiglas plate to view under the inverted microscope. The exposed mesentery is kept moist and warm by superfusion with warm phosphate-buffered saline (37 °C). Care should be taken not to traumatize the vessel or mechanically activate the endothelium. Advantages of this preparation include: (1) mesenteric vessels are thinner than carotid vessels, which makes it easier to focus light and get both bright-field and fluorescent images, (2) the ease of quantifying adhesion of single platelets, and (3) although not preferred, multiple vessels can be studied in a single mouse. Disadvantages are that vessels get traumatized easily, and it is not feasible to study older mice because of the large amount of fat around the vessels.

3. *Cremaster muscle vasculature* (Welsh et al. 2014; Falati et al. 2002): Male adult mice (8–24 weeks) are anesthetized, and the cremaster muscle is exteriorized through an incision in the scrotum. The muscle is pinned around a coverslip mounted on a miniature platform using a dissecting microscope. Care should be taken during dissection to avoid traumatizing the cremasteric vessels and disturbing the blood supply. The cremaster muscle is superfused throughout the experiment with preheated (37–38 °C) bicarbonate-buffered saline drip aerated with 5 % CO_2/95 % N_2, with the mouse body temperature maintained at 37–38 °C using a rectal probe or heated blanket. The purpose of the bicarbonate saline is to maintain the viability of the cremaster muscle and to prevent muscle spasm. Advantages of studying the cremaster muscle vasculature compared to carotid or mesentery vessels are as follows: (1) cremasteric vessels are thin, and therefore, it is relatively easy to obtain high-resolution bright-field images, (2) the cremaster muscle can be isolated in mice from 4 to 25 weeks of age, and (3) multiple mild pinpoint injuries can be induced in cremaster arterioles (usually 20–40 μm in diameter) using a pulsed nitrogen dye laser. Disadvantages include the following: (1) only male mice can be studied, (2) cremasteric arterioles and venules are too small to induce a mechanical injury, (3) it is difficult to measure flow rate using a Doppler flow probe, and (4) a laser-induced thrombus grows to maximum size in approximately 1 min and then gradually disintegrates within 2–3 min.

Methods of Induction of Injury

Several methods can be used to induce thrombus formation in vivo.

1. *Ferric chloride* (*$FeCl_3$*): $FeCl_3$ is one of the most widely used methods to induce thrombosis in vivo. $FeCl_3$ solution (5–20 %) is either directly superfused on the artery or topically applied using a filter paper (1 × 1 mm) saturated with $FeCl_3$ (5–20 %) for 3–5 min. The severity of injury and resulting time to occlusion depends on the concentration of $FeCl_3$ solution used for injury. A stable and completely occlusive thrombus generally forms within 20 min from the time 7.5 % $FeCl_3$ solution is applied. Using in vivo microscopy, it is possible to measure adhesion of single platelets beginning 3–5 min after injury and then to monitor thrombus growth kinetics and time to occlusion (Prakash et al. 2015b). Alternatively, using a Doppler flow probe, one can monitor blood flow within an artery and calculate the time to occlusion. Several studies suggest that this model is sensitive to both anticoagulant and antiplatelet drugs (Day et al. 2004, 2005; Wang et al. 2005; Pinel et al. 2004; Marsh Lyle et al. 1998). The mechanism of $FeCl_3$-induced vascular injury appears to be complex. Previously, it was shown that $FeCl_3$ causes oxidative stress with the generation of free radicals leading to lipid peroxidation that results in denudation of the endothelium leading to exposure of the subendothelium (Tseng et al. 2006; Eckly et al. 2011). In contrast, other studies have suggested that $FeCl_3$ does not result in endothelial denudation (Barr et al. 2013; Ciciliano et al. 2015). Another study suggested that $FeCl_3$-induced injury is indirect and occurs as a consequence of erythrocyte lysis (Woollard et al. 2009). A recent study suggests that $FeCl_3$ induces early aggregation of plasma proteins and blood cells, which is mediated by charge-based binding of proteins (either cell surface bound or soluble) to the Fe^{3+} ions (Ciciliano et al. 2015). Whatever the multifaceted mechanism of $FeCl_3$ is on thrombosis, this model remains a valuable tool to screen inhibitors of platelet activation and subsequent thrombus formation in vivo (Day et al. 2004, 2005; Wang et al. 2005; Pinel et al. 2004; Marsh Lyle et al. 1998).
2. *Photochemical injury with Rose Bengal* (Wilson et al. 2003; Day et al. 2004): This method requires systemic administration of photoreactive substance, Rose Bengal, followed by local transillumination of the target vessel with a green laser light (540 nm). Rose Bengal is known to accumulate in the lipid bilayer of endothelial cells (Saniabadi et al. 1995). A photochemical reaction between green light and Rose Bengal produces singlet oxygen and promotes formation of other reactive oxygen species that damage the endothelium and initiate thrombus formation. This model results in a milder injury compared to the $FeCl_3$ method, and therefore, the time to form occlusive thrombus is longer, approximately 40 min. Time to occlusion can be measured using a Doppler flow probe or by in vivo microscopy. Mice are injected typically with 10–50 mg/kg Rose Bengal using a jugular vein, femoral vein, or tail vein. Several factors are necessary to obtain reproducible results: (1) the same amount of Rose Bengal per gram should be infused in each animal; (2) laser light should be maintained at a constant distance, usually 5–6 cm, from the blood vessel; and (3) the vessel should be illuminated with the laser light before the Rose Bengal infusion is begun. This method has been used widely to study carotid artery thrombosis in mice that are genetically altered for hemostatic or fibrinolytic factors involved in thrombus formation as well as in mice with hyperlipidemia or leptin deficiency (Eitzman et al. 2000).
3. *Laser-induced injury* (Falati et al. 2002; Stalker et al. 2014): Injury is initiated in the microvasculature using a

micropoint laser ablation system. The user can obtain precise illumination of the area of interest through the microscope eyepiece or with the imaging system in real time. The wavelength of light used for illumination is in the range of 365–400 nm with maximum output from 50 to 500 μJ that can be selected by the user via a dye cell and an attenuator to adjust the energy. The power and frequency of bursts are controlled by software and can be empirically defined depending on the thickness of the vessel. Care must be taken to assure that the emission filter being used absorbs light at the frequency of the laser, to avoid stray reflections that might damage the intensifier or the camera. Thrombi can be generated either in arterioles or venules. The laser injury-induced thrombus grows to its maximum size in approximately 1 min and then gradually disintegrates in the following 2–3 min. Multiple thrombi can be generated over the course of a 60–90 min experiment either in a single vessel or multiple vessels in a single mouse.

4. *Mechanical injury* (Stockmans et al. 1991; Roque et al. 2000): Endothelial injury in the vessel is either induced using forceps or by passage of an angioplasty guide wire. In the latter case, femoral or carotid arteries are temporarily clamped, and an arteriotomy is made to introduce the angioplasty guide wire. Endothelial injury is made by rotating the wire to ensure uniform and complete endothelial denudation. After removing the wire, the arteriotomy site is ligated and platelet-vessel wall interactions can be studied using intravital microscopy.
5. *Electrolytic injury* (Cooley 2011): Injury is induced on the surface of the carotid artery with a 30 s, 3-V direct current application, touching the surface of the vessel with the blunt end of a 140-μm diameter steel needle connected to the anode, completing the circuit by contacting local subdermal tissue with the cathode. The thrombosis with the electrolytic injury is similar to $FeCl_3$ injury-induced thrombosis (Cooley 2011).

Effect of Mechanical Ventilation on Thrombosis

Some research groups mechanically ventilate mice during anesthesia in studies of experimental thrombosis. Prolonged anesthesia has been shown to be associated with respiratory acidosis and increased cerebral blood flow (Dalkara et al. 1995; Vogel et al. 2000). Lentz and colleagues have demonstrated that mice (C57BL6/J or BALB/c genetic background) anesthetized with sodium pentobarbital (70–90 mg/kg body weight, intraperitoneally) without mechanical ventilation and exhibit hypercapnia, acidosis, and increased carotid artery blood flow with prolonged times to complete occlusion after photochemical injury (Wilson et al. 2003). Adequate mechanical ventilation with room air and supplemental oxygen (80 breaths/min, tidal volume 0.2–0.5 mL) prevented hypercapnia and acidosis, normalized carotid artery blood flow, and shortened the time to carotid artery occlusion. However, it was not shown and remains uncertain whether the effect of anesthesia on acidosis and carotid blood flow is a general phenomenon or depends on the type and dose of anesthesia used for the study. Although this study was done in the context of carotid artery thrombosis, the possibility that mechanical ventilation may influence the susceptibility to thrombosis in other vascular beds, including the femoral artery, mesenteric vessels, and cremaster muscle microcirculation, cannot be ruled out. Nevertheless, this study clearly suggests that to study thrombosis in vivo, it is advisable to mechanically ventilate mice in order to maintain physiological conditions, particularly during prolonged periods of anesthesia.

Methods of Visualizing and Quantifying Various Components of Thrombi

There are several approaches that can be used to visualize various components of the thrombus in real time. For multichannel microscopy, platelets and plasma proteins can be labeled simultaneously with different fluorochromes. Care should be taken to account for tissue autofluorescence and crossover (bleeding) of signals from one fluorochrome to another. Common fluorochromes used are Alexa 350, 448, 567, and 660. The best choice is to use two fluorochromes that are wider in excitation wavelength. For example, for two-channel simultaneous imaging, it is better to use Alexa 448 with Alexa 660. In case of three-channel imaging, one can use Alexa 350, 448, and 660. Tail vein, femoral vein, and jugular vein are the obvious choices to infuse fluorescent antibodies, reagents, and drugs into the circulation.

To quantitatively assess thrombus growth in real time, the following parameters can be evaluated:

(1) Single platelet-vessel wall interactions determined as the number of fluorescent platelets that are deposited on the injured vessel wall segment during 1 min (beginning 2–3 min after injury)
(2) The time required for formation of the first thrombus larger than 30–50 μm
(3) Thrombus growth rate
(4) Thrombus stability, determined by measuring the number of thrombi of diameter larger than 30–50 μm embolizing before vessel occlusion
(5) Time to occlusion (i.e., the time required for blood to stop flowing for 30 s)
(6) The site of vessel occlusion (i.e., whether the occlusion occurs at the site of injury or downstream)

To quantify all these parameters, it is important to label platelets with fluorescent dyes or Alexa-labeled antibodies

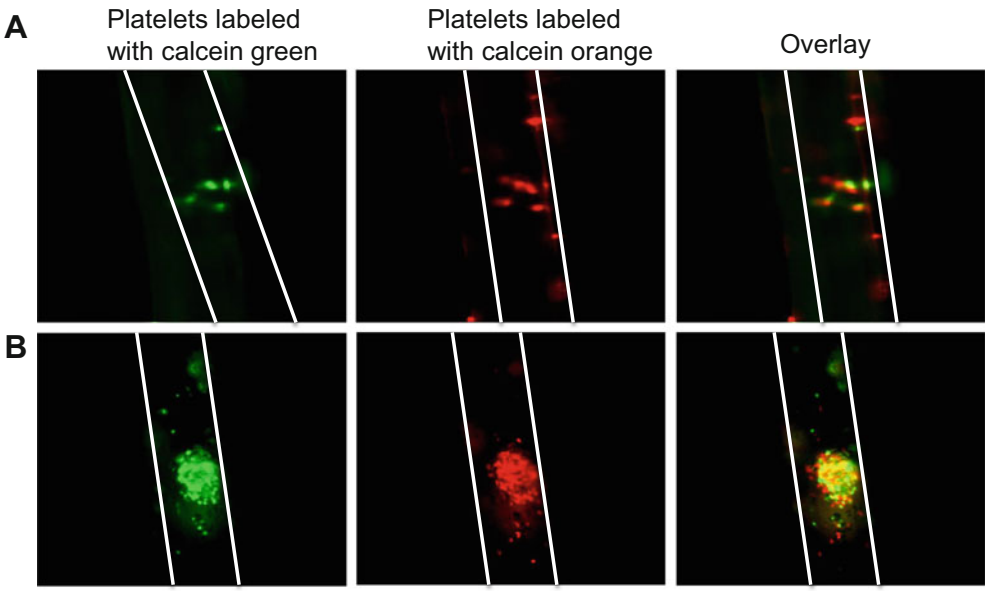

Fig. 1 In vivo imaging of platelets/thrombosis in wild-type mouse. Injury was made in mesenteric arterioles (100–125 μM) using 5 % $FeCl_3$. Platelets were labeled ex vivo with calcein green or calcein orange and infused in the recipient mouse. (**a**) Microphotographs show single platelet adhesion following 3–4 min after injury. (**b**) Microphotographs show platelet thrombi following 10–12 min after injury. *White line* delineates vessel

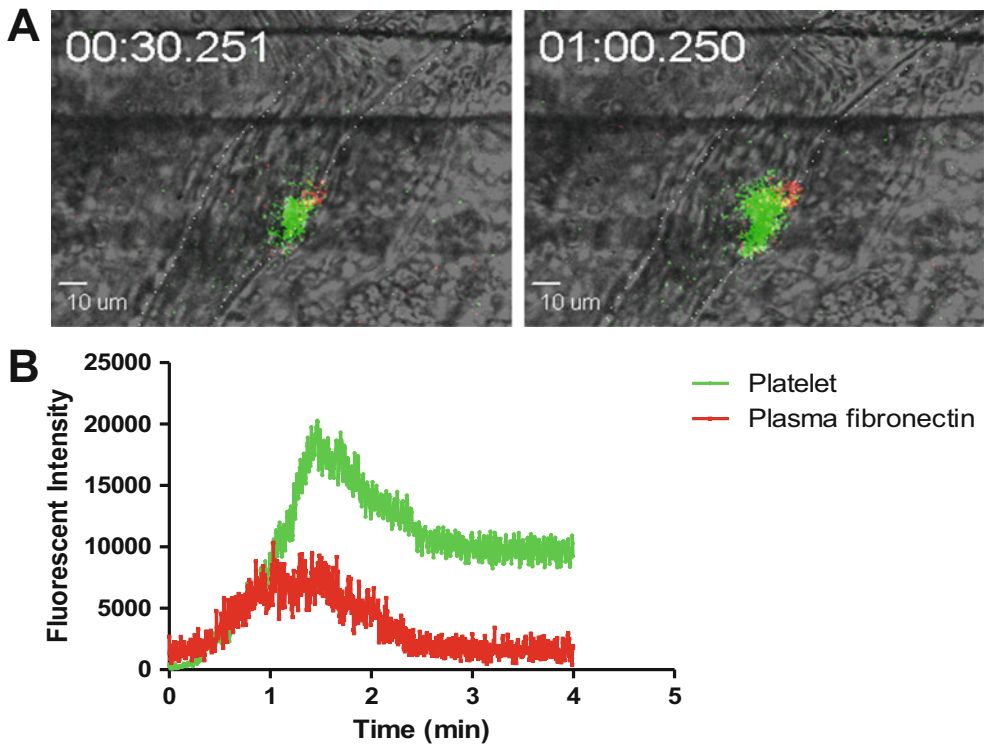

Fig. 2 In vivo imaging of thrombus in laser-induced cremaster arterial thrombosis model. (**a**) The platelets are labeled with anti-GPIb antibody conjugated with DyLight 488 (*green*) and plasma fibronectin conjugated with AF647 (*red*). (**b**) Kinetics of platelet accumulation and plasma fibronectin deposition in growing thrombus. Figure provided by Dr. Heyu Ni

that are specific for platelets, taking care that the antibodies do not cause thrombocytopenia or interfere with important platelet functions. Platelets can be isolated from donor mice and fluorescently labeled with calcein AM (2.5 μg/mL, Molecular Probes) ex vivo and infused into recipient mice of the same genotype through a tail vein at a concentration of 2.5×10^9 platelets/kg body weight (Fig. 1) (Denis et al. 1998; Chauhan et al. 2006). The use of fluorescently labeled anti-GPIbβ (Emfret analytics) or anti-CD41 ($F(ab)_2$ fragment) antibodies can also be used to label endogenous platelets in vivo (Fig. 2) (Dubois et al. 2007; Falati et al. 2002; Stalker et al. 2014; Welsh et al. 2014). It is important to utilize the correct isotype-matched controls and to pay attention to the relative fluorescence labeling of each antibody. The use of $F(ab)_2$ fragments is preferable, to minimize the clearance of platelets via Fc receptors, which may result in moderate to severe thrombocytopenia.

To measure other parameters of platelet activation in vivo, one can monitor calcium mobilization and/or P-selectin expression. To quantify cytosolic calcium

mobilization in platelets, Fura 2-AM, a calcium ion-binding fluorochrome, is used (Dubois et al. 2007; Panicot-Dubois et al. 2007). Washed platelets are labeled with Fura 2-AM and infused into a recipient mouse of the same genotype. Calcium binding to Fura-2 results in a shift in the absorbance of the dye from 380 to 340 nm. Fura 2-AM-loaded platelets in growing thrombus are visualized after excitation at 380 nm, and calcium mobilization during thrombus formation is visualized after excitation at 340 nm (Dubois et al. 2007; Panicot-Dubois et al. 2007). To determine the kinetics of calcium mobilization within a growing thrombus, medium integrated fluorescent intensities are compared. To label P-selectin, fluorescent anti-CD62P is used. Similarly, tissue factor and fibrin can be quantified in real time within the growing thrombus using appropriate antibodies and their respective controls (Falati et al. 2002).

Recent studies from the Brass laboratory have shown that it is possible to measure intra-thrombus molecular transport rates in real time during thrombus formation (Welsh et al. 2014; Stalker et al. 2014). In this method, albumin is labeled with caged fluorescein (cAlb) molecules that become stably fluorescent only after being uncaged by exposure at 405 nm. Once uncaged, cAlb in the thrombus is measured as decay in mean fluorescence intensity over time (Stalker et al. 2014; Welsh et al. 2014). The same group has also developed a thrombin activity sensor mTHS-Ab (Welsh et al. 2012). This sensor is a thrombin substrate consisting of a fluorescence resonance energy transfer-based fluorogenic peptide linked to an anti-mCD41 antibody targeted to the platelet surface. Cleavage of the peptide by thrombin releases a quencher, allowing an increase in fluorescence signal, which represents the spatial distribution of cumulative thrombin activity (Welsh et al. 2014).

Conclusion

Hemostasis and thrombosis are complex processes, which require blood cellular components as well as plasma components. The main strength of in vivo imaging is that it provides information about these processes in live animals that cannot be obtained by in vitro or ex vivo methods. A clear example is the response of the vasculature to platelets under conditions of physiological and pathological stimuli. Although in vitro or ex vivo approaches such as flow chambers, aggregation, and spreading assays provide information about platelet activation, in vivo imaging enables determining the role and relative contribution of several blood components including platelets and plasma proteins in a physiological context in an intact animal. In the past years, the data generated from in vivo imaging has been instrumental in understanding the mechanisms of thrombus formation and designing effective antithrombotic agents.

Take Home Messages

- In vivo imaging has increased our understanding of the mechanism of thrombus formation in the physiological context.
- The information provided in this chapter may assist in designing and testing the efficacy of antithrombotic drugs in preclinical models.

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Monitoring of Antiplatelet Therapy

Udaya S. Tantry, Aung Myat, and Paul A. Gurbel

Abstract

The variability of response to antiplatelet agents and its potential relation to the occurrence of recurrent ischemic events have stimulated the concept of antiplatelet therapy monitoring over the last decade. This has been driven further by the development of user-friendly point-of-care methods to assess platelet reactivity to adenosine diphosphate. Large observational studies have established an independent relation between the results of point-of-care platelet function testing and adverse clinical outcomes, particularly in patients undergoing coronary artery stenting. Furthermore, the concept of a "therapeutic window" for $P2Y_{12}$ receptor reactivity has been proposed, with an upper threshold associated with ischemic events and a lower threshold associated with bleeding. Prospective randomized trials have, however, failed to demonstrate that tailored antiplatelet therapy based on point-of-care assessment of platelet function is effective in reducing ischemic event occurrences. In the absence of robust prospective evidence to support a personalized approach, decisions regarding antiplatelet therapy rest on patient comorbidity, the large body of observational data, national and international guidelines, and recognition of the fundamental importance of platelet physiology in causing catastrophic events in those with high-risk coronary artery disease.

Introduction

Overwhelming evidence exists to support the theory that platelet-rich thrombus generation at the sites of plaque rupture and vessel wall erosion is a primary process causing ischemic events in patients with coronary artery disease. Following spontaneous plaque rupture in acute coronary syndrome (ACS) and induced rupture during percutaneous coronary intervention (PCI), platelets adhere to the injured vessel wall and undergo activation that is followed by release of the secondary agonists thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP). Although TxA_2 and ADP act synergistically during platelet aggregation, the ADP–$P2Y_{12}$ receptor interaction plays a central role in sustaining the activation of glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) receptors by amplifying the response to agonists, leading to stable platelet-rich thrombus generation at the site of vessel wall injury. A vicious cycle occurs in which coagulation factors are formed on the activated platelet surface, generating more thrombin and further enhancing platelet activation and coagulation processes. The platelet-rich thrombus is further stabilized by fibrin mesh formation that is simultaneously generated by thrombin through the coagulation cascade (Gurbel et al. 2004) (Fig. 1). These observations position the platelet as a "nidus of evil" and are the rationale for dual signaling pathway blockade with cyclooxygenase 1 (COX-1) and $P2Y_{12}$ receptor inhibitors in patients with ACS and those undergoing PCI (Gurbel and Tantry 2010).

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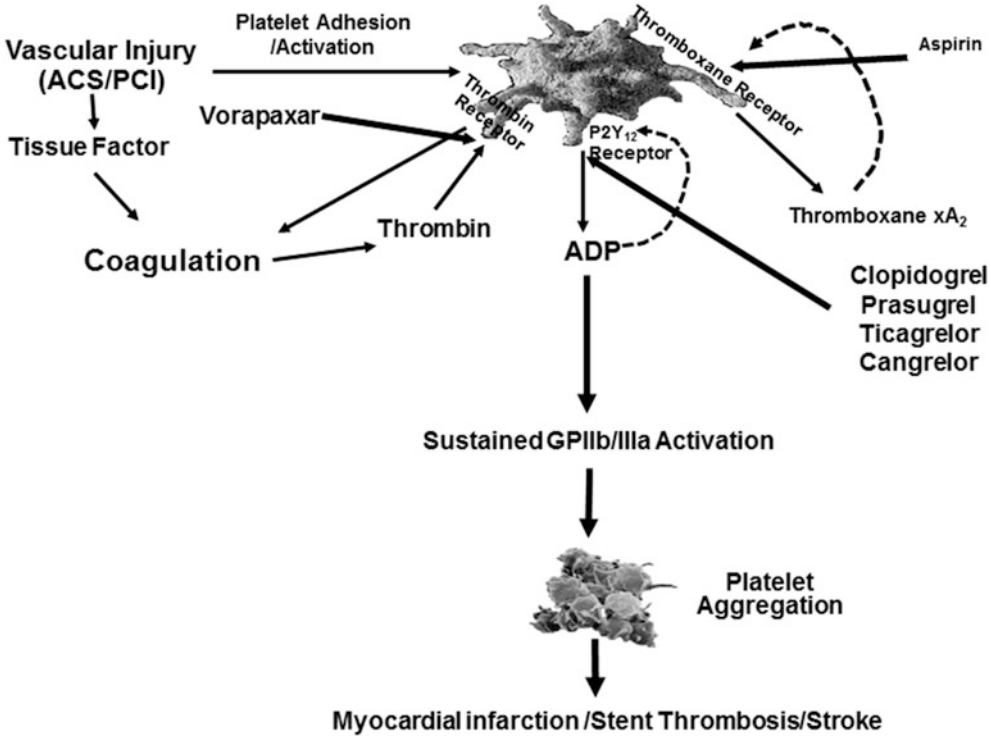

Fig. 1 Role of different platelet receptors in thrombotic events. Following vascular injury, platelets adhere to the injury site and undergo activation. Platelet activation results in the release of three important platelet agonists: thromboxane A_2 (TxA_2), adenosine diphosphate (ADP), and thrombin. The thrombin–protease activated receptor-1, TxA_2–thromboxane receptor, and ADP–$P2Y_{12}$ receptor pathways amplify the response to platelets, resulting in sustained platelet aggregation via activated $\alpha_{IIb}\beta_3$ receptor. The ADP–$P2Y_{12}$ interaction plays a central role. Formation of occlusive platelet-rich thrombus formation at the site of plaque rupture is mainly responsible for the occurrence of arterial thrombotic events such as myocardial infarction, stent thrombosis, and stroke

Current guidelines recommend a non-monitored or universal "one-size-fits-all" approach, despite the fact that the pharmacodynamic effect of $P2Y_{12}$ inhibitors is widely variable, resulting in both poor and enhanced responders to therapy (Bonello et al. 2010; Aradi et al. 2014; Tantry et al. 2013). There has been a longstanding reluctance to assess platelet function because of the potential introduction of artifacts by laboratory methods, incomplete reflection of the actual in vivo thrombotic process, and failure to establish unequivocally a causal relation between the results of the test and thrombotic event occurrence (Hirsh et al. 1981). In the last decade, understanding of platelet receptor physiology has markedly improved and more potent $P2Y_{12}$ receptor blockers that can overcome some of the limitations of clopidogrel have been developed. The introduction of more user-friendly platelet function assays that can reliably determine the antiplatelet effect of clopidogrel have spurred renewed interest in antiplatelet therapy monitoring (Bonello et al. 2010; Aradi et al. 2014; Tantry et al. 2013).

Resistance to Dual Antiplatelet Therapy

Despite appropriate dual antiplatelet therapy (DAPT), a significant percentage of patients (~10 %) continue to suffer major adverse cardiovascular and cerebrovascular events following ACS and coronary stenting (Bonello et al. 2010; Aradi et al. 2014; Tantry et al. 2013; Hirsh et al. 1981). Importantly, late stent thrombosis, especially in the era of drug-eluting stents, can occur in 1–2 % of patients and the rate is reported to increase by 0.3–0.6% each year (Mauri et al. 2014). This has lead to the postulation that those individuals suffering recurrent events may represent a cohort of patients who have a less than adequate response to antiplatelet therapy.

The term "antiplatelet resistance" is controversial in this context because it has been used to encompass both failure of the antiplatelet agent to achieve its intended pharmacodynamic effect and also failure to prevent the clinical condition for which it is intended. Because the pathophysiology of

atherothrombosis involves a complex interplay between platelet function, thrombosis, inflammation, innate vascular biology, and changing hemodynamics, no single class of agent can be expected to abolish all ischemic events completely. Furthermore, a patient may have the appropriate platelet response to a given therapy but have recurrent events mediated by non-platelet factors. For these reasons, the term "antiplatelet resistance" is meaningful only when inadequate pharmacodynamic response, as indicated by a specific laboratory assay, is associated with continued occurrence of ischemic events despite adequate duration of antiplatelet therapy, dosing, and compliance.

Importance of Monitoring of Aspirin Responsiveness

Earlier studies attempted to demonstrate aspirin nonresponsiveness/resistance using laboratory methods and relate the results to clinical outcome (Gum et al. 2003; Chen et al. 2007). Laboratory methods, including point-of-care tests, use agonists such as ADP, collagen, shear stress, and epinephrine to stimulate platelets and indicate aspirin responsiveness. These methods do not exclusively indicate the level of COX-1 activity, the target of aspirin antiplatelet activity, and are considered COX-1 nonspecific methods. The optimal definition of resistance or nonresponsiveness to aspirin is the demonstration of residual activity of the COX-1 enzyme. Measurement of arachidonic acid-induced platelet aggregation is the most widely used method for indicating COX-1 activity (COX-1-specific methods) (Tantry et al. 2009; Gurbel et al. 2007). There is wide variability in values for the prevalence of aspirin resistance (<1–57 %) as a result of differences in ex vivo methods and in criteria used to define aspirin resistance. However, it has been clearly established that in the presence of even low-dose aspirin therapy the prevalence of aspirin resistance, as indicated by COX-1 activity, is rare (<5 %). Reports of a higher prevalence of aspirin resistance have been attributed to COX-1 nonspecific methods, noncompliance, or inadequate doses of aspirin in selected patients (Tantry et al. 2005, 2009; Gurbel et al. 2007). The relation of aspirin resistance (based on the VerifyNow assay) to ischemic outcome was not demonstrated in a recent large scale study (Stone et al. 2013). Moreover, in two large clinical studies, high levels of urinary 11-dehydro-thromboxane B_2 (a stable metabolite of TxA_2), as measured by enzyme-linked immunoassay, were shown to be associated with heightened risk of occurrence of a cardiovascular ischemic event (Eikelboom et al. 2002, 2008). However, a reliable and specific laboratory method for identifying aspirin resistance has not yet been uniformly accepted by investigators. Other than in research trials, it is not currently recommended to test for aspirin resistance in patients or to change therapy on the basis of such findings (Michelson et al. 2005).

Importance of Monitoring of $P2Y_{12}$ Receptor Blocker Responsiveness

A key development in the field of platelet function assessment occurred in 1962 when a quantitative ex vivo method to measure platelet function, conventional/light transmittance aggregation (LTA), was developed in the Gustav Born laboratory. Born demonstrated that platelets underwent aggregation following the addition of ADP and that it was possible to record a decrease in optical density over time that was proportional to the concentration of platelets in plasma (Born 1965). LTA, even though considered the gold standard assay for assessment of platelet function, is labor-intensive, requires expert personnel, involves centrifugation steps, and can be subject to interlaboratory differences. Two different point-of-care whole blood assays, the VerifyNow $P2Y_{12}$ assay (Accriva Diagnostics, San Diego, California) and the Multiplate Analyzer (Dynabyte Informationssysteme, Munich, Germany) (both employing ADP as the agonist) have been developed to measure platelet reactivity. The VerifyNow $P2Y_{12}$ assay is a turbidimetric assay that measures aggregation of platelets to fibrinogen-coated beads in whole blood. The Multiplate Analyzer allows measurement of platelet function in whole blood using impedance aggregometry, requires no centrifugation step, and can be used for a variety of applications such as monitoring of antiplatelet therapy and assessment of perioperative platelet function disorders in a near-patient environment. In addition, whole blood thrombelastography with platelet mapping can be used to assess $P2Y_{12}$ receptor reactivity. A point-of-care thrombelastograph known as TEG6s (Haemonetics, Braintree, MS) has also become recently available. The vasodilator-stimulated phosphoprotein phosphorylation (VASP-P) assay, based on flow cytometry, is more specific for assessment of the $P2Y_{12}$ receptor response. A more user-friendly, enzyme-linked immunoassay test is also now available (Fig. 2).

In an earlier prospective study, clopidogrel response variability and resistance was demonstrated by measuring ADP-induced platelet aggregation by LTA and expression of p-selectin and activated $\alpha_{IIb}\beta_3$ by flow cytometry serially for 30 days following stenting and administration of a 300 mg load/75 mg per day maintenance dose of clopidogrel. In this study, about 30 % of patients were resistant to clopidogrel at days 1 and 5 post-stenting, and 15 % were resistant at day 30. "Resistance" in these patients was defined as ≤10 % absolute difference between maximal pre- and post-treatment platelet aggregation induced by 5 μM ADP (Gurbel et al. 2003). Since then, numerous studies have been conducted worldwide to demonstrate a link between high platelet reactivity (HPR) to

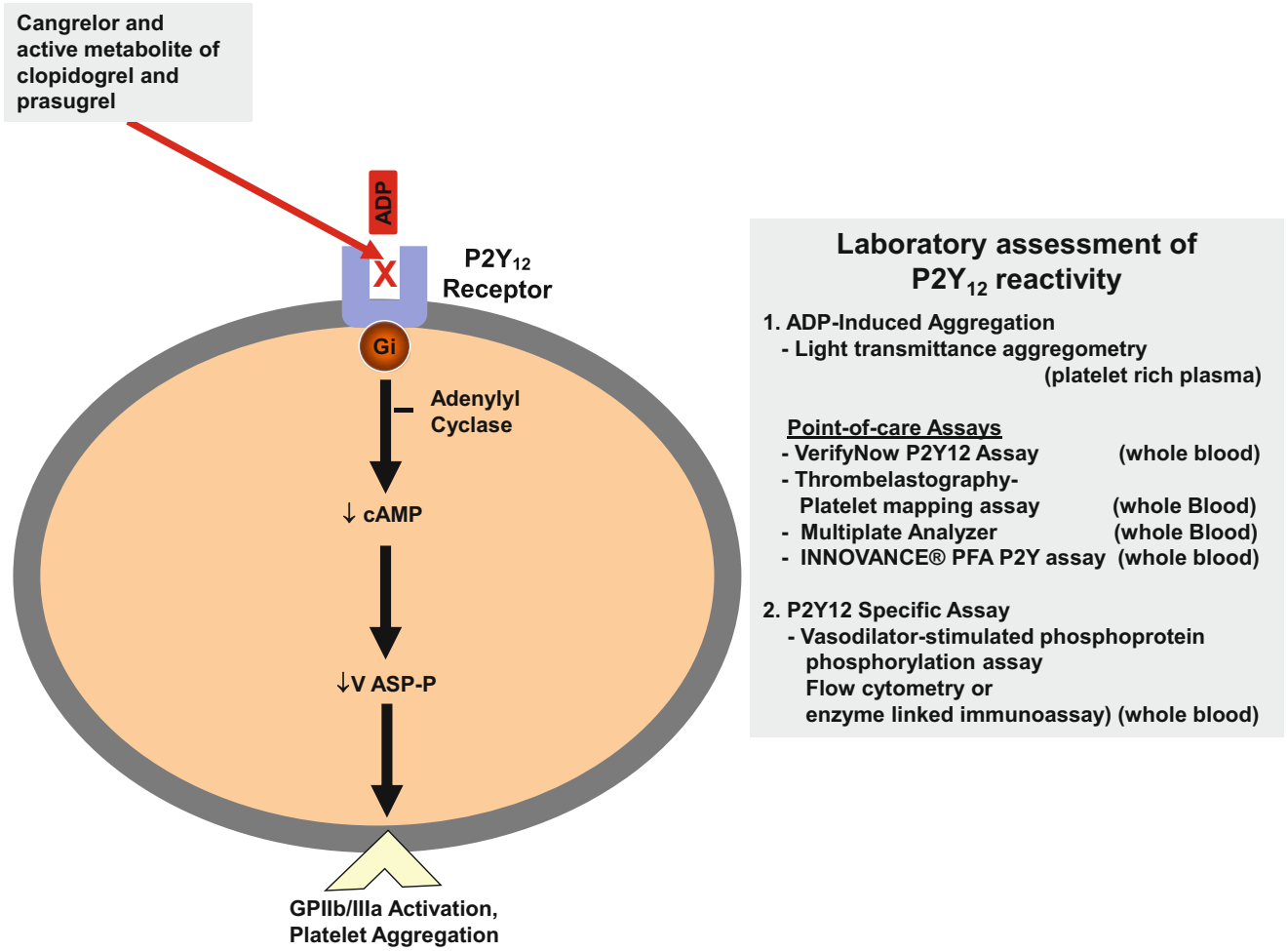

Fig. 2 Laboratory assessment of $P2Y_{12}$ receptor reactivity

ADP and ischemic outcomes. Various laboratory methods have been used, such as conventional aggregation, flow cytometry to measure P-selectin and activated $\alpha_{IIb}\beta_3$ receptor expression and vasodilator-stimulated phosphoprotein phosphorylation levels, and the point-of-care methods VerifyNow $P2Y_{12}$ assay, platelet mapping with thrombelastography, and the Multiplate Analyzer. The results of these studies have provided the strongest rationale for ex vivo quantification of the intensity of the ADP–$P2Y_{12}$ interaction in patients treated with $P2Y_{12}$ receptor blockers (Gurbel and Tantry 2012).

Relation of Ex Vivo Platelet Function Measurement to Clinical Outcome

Small early studies suggested that ischemic event occurrence was not linearly related to on-treatment platelet reactivity but instead occurred above a moderate level of platelet reactivity to ADP. The relation between an upper threshold of on-treatment platelet reactivity to post-PCI ischemic events was first demonstrated in the study PREPARE POST-STENTING (platelet reactivity in patients and recurrent events post-stenting) (Gurbel et al. 2005a). Similarly, ADP-induced platelet aggregation was shown to correlate with stent thrombosis and with increased ischemic event occurrence within 12 months of PCI (Gurbel et al. 2005b; Bliden et al. 2007). Subsequent studies used receiver operating characteristic (ROC) curve analysis to define an optimal cutoff for on-treatment platelet reactivity for predicting thrombotic risk. In 2010, a consensus statement proposed cutoff values (based on ROC curve analysis) for different platelet function assays to be used in future studies of personalized antiplatelet therapy (Bonello et al. 2010).

Personalized Antiplatelet Therapy Trials

Prospective, albeit small, tailored antiplatelet therapy studies provided initial evidence that HPR might not just be useful as a diagnostic marker but also as a modifiable risk factor for

post-PCI ischemic event occurrence. In two trials, tailored incremental loading doses of clopidogrel before PCI overcame HPR and were effective in reducing the occurrence of major adverse cardiac events within 30 days of PCI (Bonello et al. 2008, 2009). Similarly, two other studies demonstrated that administration of selective $\alpha_{IIb}\beta_3$ receptor blocker to PCI patients who demonstrated HPR following clopidogrel loading was effective in reducing subsequent periprocedural as well as long-term (1 year) ischemic outcomes (Campo et al. 2010; Cuisset et al. 2008). These studies were the first to suggest that the cutoff value used to identify PCI-treated patients at increased risk of thrombotic event occurrence were also useful for individualizing therapy and led to an improved outcome.

The first large-scale investigation of personalized antiplatelet therapy, conducted in stable patients treated with coronary stents, was GRAVITAS (gauging responsiveness with a VerifyNow assay: impact on thrombosis and safety) ($n = 2214$). In GRAVITAS, patients with HPR after treatment with clopidogrel were randomly assigned to continue standard clopidogrel dosing or to be treated with a higher clopidogrel dose. The 6-month composite ischemic event occurrence rate was identical (2.3 %) for the high-dose clopidogrel treatment arm (600 mg extra loading dose given the day after stenting, followed by a 150 mg daily maintenance therapy) and the standard dose treatment arm (75 mg daily maintenance therapy) (Price et al. 2011). Potential explanations for the neutral observation of GRAVITAS have been proposed and include suboptimal efficacy of high-dose clopidogrel to overcome HPR, a cutoff for HPR that was too high, and enrollment of predominantly low-risk patients with stable coronary artery disease, which resulted in a very low event rate and undersizing of the study (Gurbel and Tantry 2011).

The study TRIGGER-PCI (testing platelet reactivity in patients undergoing elective stent placement on clopidogrel to guide alternative therapy with prasugrel) ($n = 212$) was conducted in elective stable PCI patients, excluding non-ST-segment elevation and ST-segment-elevation myocardial infarction (NSTEMI and STEMI, respectively) patients. A 10 mg daily dose of prasugrel was used in the active arm to treat patients with HPR and was highly effective in reducing its prevalence; only ~6 % of patients had HPR after 90 days of prasugrel therapy. However, the study was terminated early because of futility. There was only one occurrence of the primary end point among 236 patients who completed 6 months of follow-up. In addition, ~30 % of the enrolled patients declined randomization after being identified as having HPR (Trenk et al. 2012).

In the study ARCTIC (assessment by a double randomization of a conventional antiplatelet strategy versus a monitoring-guided strategy for drug-eluting stent implantation and of treatment interruption versus continuation one year after stenting) ($n = 2440$), patients who had undergone successful coronary stenting were randomly assigned to a strategy of platelet-function monitoring, with adjustment of antiplatelet therapy in patients who had a poor response to clopidogrel (>208 $P2Y_{12}$ reaction units as assessed by VerifyNow $P2Y_{12}$ assay) or to a conventional strategy without monitoring and drug adjustment. The 1-year primary composite end point of death, myocardial infarction, stent thrombosis, stroke, or urgent revascularization was not different in the monitoring arm compared with the conventional arm (34.6 % versus 31.1 %, hazard ratio 1.13; $p = 0.10$). The prevalence of patients with ACS was low (27 % NSTE-ACS compared with 73 % patients with stable coronary artery disease). STEMI patients, a group at high risk for early atherothrombotic events, were excluded. HPR was mainly treated as in the GRAVITAS trial, with double-dose clopidogrel. Prasugrel, a superior alternative to double-dose clopidogrel for overcoming HPR, was only administered to ~10 % of patients (Collet et al. 2012).

These randomized studies all used the VerifyNow assay to assess platelet reactivity to ADP and to identify HPR. The study included mostly low-risk patients undergoing PCI and had resultant low event rates irrespective of platelet reactivity. Given the post-discharge event rates observed in ARCTIC, it was estimated that 17,540 patients would be necessary to refute the utility of personalized therapy (De Caterina et al. 2013). The results of all these studies also suggest that high-dose clopidogrel is not an optimal strategy for overcoming HPR and improving clinical outcomes.

Although a major risk factor for post-PCI thrombotic event occurrence, HPR is not the sole factor responsible for these events. In contrast, the absence of HPR is the best reassurance thus far for a low likelihood of future ischemic events. The HPR cutoff values reported in many studies are associated with high negative predictive values and low positive predictive values. However, given the overall low prevalence of thrombotic events in these studies, the low positive predictive values and high negative predictive values are understandable. Other factors, including demographic, clinical, and angiographic factors, must be taken into consideration for optimal identification of patients at greatest risk. Along this line, recent studies have suggested that adding clinical variables and genotype to platelet reactivity measurements (a combined risk factor) could improve risk prediction (Geisler et al. 2008; Fontana et al. 2010).

Therapeutic Window Concept for $P2Y_{12}$ Receptor Reactivity

In addition to the upper threshold for ischemic risk (i.e., HPR) described earlier, the relationship between low platelet reactivity and bleeding was demonstrated in small

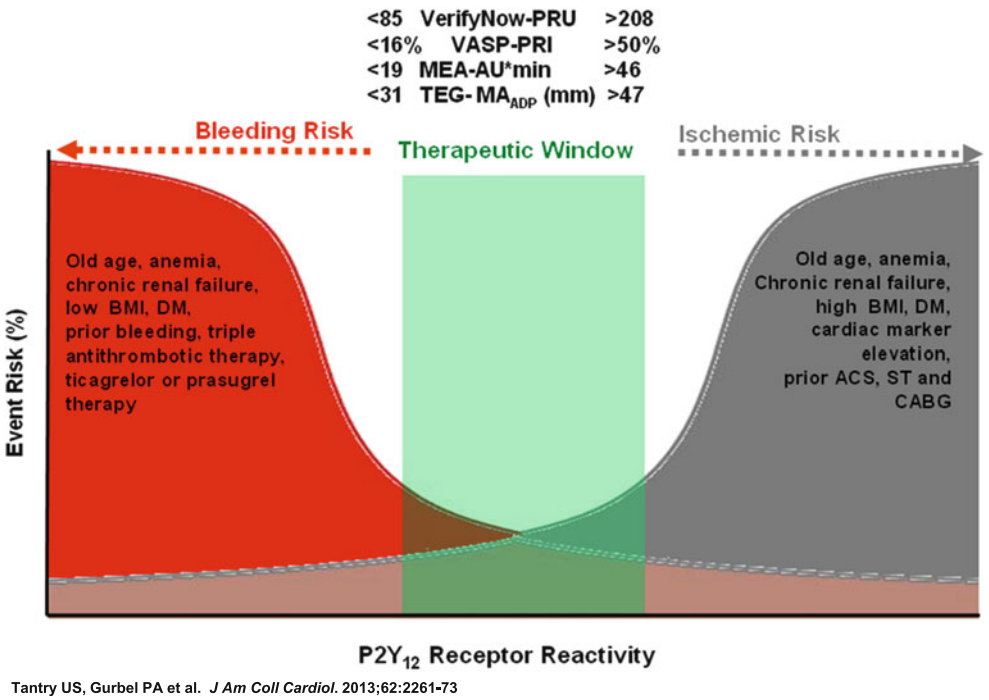

Fig. 3 Evidence for $P2Y_{12}$ receptor reactivity associated with post-PCI ischemic and bleeding events. Adapted from Tantry et al. (2013). Similar to previously proposed high on-treatment platelet reactivity to ADP associated with post-PCI ischemic events, recent data suggest that low on-treatment platelet reactivity to ADP is associated with a higher risk of bleeding. Cutoff values for both ischemic and bleeding events based on various platelet function assays are shown. Both ischemic and bleeding events are influenced by various demographic variables, as indicated. *ACS* acute coronary syndromes, *AU* arbitrary aggregation units, *BMI* body mass index, *CABG* coronary artery bypass graft, *DM* diabetes mellitus, *MA* maximum amplitude, *MEA* multiplate analyzer, *PRI* platelet reactivity index, *PRU* $P2Y_{12}$ reaction units, *ST* stent thrombosis, *TEG* thrombelastography, *VASP* vasodilator stimulated phosphoprotein-phosphorylation

Table 1 Platelet reactivity cutoff associated with ischemic and bleeding events (therapeutic window)

	Cutoff associated with ischemic event occurrences	Cutoff associated with bleeding event occurrences
VerifyNow PRU Assay (PRU)	>208 (Campo et al. 2011)	<85 (Campo et al. 2011)
Multiplate Analyzer ADP-induced aggregation (AU × min)	>46 (Sibbing et al. 2010)	<19 (Sibbing et al. 2010)
Thrombelastography Platelet Mapping Assay ADP-induced platelet-fibrin clot strength (mm)	>47 (Gurbel et al. 2010)	<31 (Gurbel et al. 2010)
VASP-PRI (%)	≥50 (Bonello et al. 2012)	<16 (Bonello et al. 2012)

ADP adenosine diphosphate, *AU* arbitrary aggregation units, *PRU* $P2Y_{12}$ reaction units, *VASP-PRI* vasodilator-stimulated phosphoprotein phosphorylation-platelet reactivity index

translational research studies. The concept of a "therapeutic window" for $P2Y_{12}$ receptor reactivity associated with both ischemic event occurrence (upper threshold) and bleeding risk (lower threshold) has been proposed. A consensus document highlighting the above observations, with a therapeutic window concept with updated cutoffs for HPR and low platelet reactivity in the setting of $P2Y_{12}$ inhibitor therapy, has been published (Fig. 3, Table 1). This approach is more meaningful when treating patients with potent $P2Y_{12}$ receptor blockers that are known to be associated with increased incidences of bleeding (Stone et al. 2013; Campo et al. 2011; Sibbing et al. 2010; Gurbel et al. 2010; Bonello et al. 2012).

The study ADAPT-DES (platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents) was a multinational prospective registry of 8582 patients (~50 % of patients with ACS). HPR was defined as >208 PRU and was independently associated with a twofold increased risk of 2-year definite/probable stent thrombosis (hazard ratio 1.84, $p = 0.009$) and inversely correlated with major bleeding (hazard ratio 0.82, $p = 0.02$), with a trend towards all-cause death (hazard ratio 1.27, $p = 0.06$). In a later report, the authors further analyzed ADAPT-DES platelet reactivity data categorized in quintiles (a continuous variable rather than the dichotomous variable used with HPR) in relation to clinical

outcomes. They found that PRU was associated in a monotonic fashion with stent thrombosis and that stent thrombosis was independently associated with highest quintile PRU. All-cause mortality was only associated with higher PRU in an unadjusted analysis (Stone et al. 2013; Kirtane 2013).

Personalized Antiplatelet Therapy in Patients Undergoing Surgery

The major rationale for 5–7 days discontinuation of $P2Y_{12}$ receptor inhibitor treatment recommended by the guidelines in patients undergoing coronary artery bypass grafting (CABG) was to allow platelet function recovery, thereby avoiding excessive perioperative bleeding. In a small prospective study, it was demonstrated that clopidogrel-treated patients undergoing on-pump CABG for the first time had similar bleeding (24-h chest tube output and number of transfused red blood cells) to clopidogrel-naïve patients when surgery was scheduled on the basis of a preoperative assessment of platelet reactivity to ADP. Moreover, the individualized timing of surgery reduced the overall preoperative waiting period by ~50 % as compared with the time recommended in the guidelines. Preoperative platelet reactivity to ADP was measured by thrombelastography (TEG with platelet mapping). Surgery in patients treated with clopidogrel was scheduled within 24 h of the last dose of clopidogrel in those with a maximum amplitude (MA_{ADP}) of >50 mm, within 3–5 days of the last dose in those with an MA_{ADP} of 35–50 mm, and 5 days after the last dose in those with an MA_{ADP} of <35 mm (Mahla et al. 2012).

Guidelines

In 2012, updated American and European practice guidelines included a Class IIb recommendation for platelet function testing to facilitate the choice of $P2Y_{12}$ receptor inhibitor in selected high-risk patients treated with PCI (Jneid et al. 2012; Hamm et al. 2011; Levine et al. 2011). In the 2012 update to the Society of Thoracic Surgeons guideline on use of antiplatelet drugs in patients having cardiac and non-cardiac operations, there is a Class IIa recommendation for platelet function testing in clopidogrel-treated patients to shorten the preoperative waiting period (Ferraris et al. 2012). Similarly, there is a Class IIb recommendation to consider platelet function testing for shortening the time interval to CABG following $P2Y_{12}$ inhibitor discontinuation in the 2015 European Society of Cardiologists guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation (Roffi et al. 2016).

Conclusions

Platelet activation and aggregation resulting in thrombus generation at the site of plaque rupture is a primary underlying factor responsible for the development of ischemic events in patients with cardiovascular disease. The development of user-friendly methods for assessing platelet reactivity to ADP has resulted in a large body of data supporting a strong association between the results of ex vivo platelet function testing and clinical event occurrence. Based on observational studies conducted in thousands of patients, international consensus accepts that HPR is a major risk factor for post-PCI ischemic event occurrence. However, large prospective trials utilizing the VerifyNow assay have thus far failed to demonstrate that personalized antiplatelet therapy is effective in reducing ischemic events. Potential explanations for the neutral results could be related to the assay itself, the lower risk of the patient group studied, the potency of the $P2Y_{12}$ inhibitor chosen to address HPR, the number of patients enrolled, and that HPR may not be a modifiable risk factor. The HPR cutoff values have high negative predictive value for thrombotic event occurrence, but the positive predictive value is low. The latter is a result of very low thrombotic event rate and the fact that although HPR is a major determinant of thrombotic event occurrences, it is not associated with all-cause mortality. Moreover, all-cause mortality is dependent on multiple factors in addition to HPR. Therefore, although HPR is not a stand-alone risk factor, it should be part of the risk algorithm along with biomarker testing (akin to CRUSADE or GRACE risk scores) to improve risk prediction and facilitate personalization of antiplatelet therapy.

In addition, atherothrombosis is a dynamic process and it is unclear whether the platelet reactivity phenotype and the hypercoagulability state remain stable over time. The results of ADAPT-DES and PEGASUS studies suggest that although long-term ticagrelor and prasugrel treatment can improve net outcomes in patients with a low bleeding risk, there is continued risk of recurrent ischemic events in selected patients even after 3–4 years of DAPT (Stone et al. 2013; Bonaca et al. 2016). The optimal treatment of these high-risk patients may require assessment of markers that better identify the presence of vulnerable vasculature and vulnerable blood, including continued monitoring of $P2Y_{12}$ receptor reactivity to improve patient selection (Gurbel and Tantry 2016).

The concept of a therapeutic window for $P2Y_{12}$ receptor reactivity associated with both ischemic event occurrence (upper threshold) and bleeding risk (lower threshold) has been proposed. The ongoing study TROPICAL ACS (testing responsiveness to platelet inhibition on chronic antiplatelet treatment for acute coronary syndromes)

($n = 2600$) aims to evaluate an approach guided by platelet function (assessed by impedence aggregometry using the Multiplate Analyzer) for personalizing antiplatelet therapy in ACS patients treated with PCI. At this time, in the absence of strong prospective evidence to support personalized antiplatelet therapy, clinical decision-making about antiplatelet therapy rests on the large body of observational data and the fundamental importance of platelet physiology in catastrophic event occurrence in patients with high-risk coronary artery disease.

Take Home Message

1. The central role of platelets provides the rationale for dual signaling pathway blockade with COX-1 and $P2Y_{12}$ receptor inhibitors in patients with ACS and those undergoing PCI.
2. High on-treatment platelet reactivity to ADP in the presence of $P2Y_{12}$ receptor blockade is an independent risk factor for ischemic event occurrence in patients undergoing coronary artery stenting.
3. The above observation is the strongest rationale for ex vivo quantification of the intensity of the ADP–$P2Y_{12}$ interaction in patients treated with $P2Y_{12}$ receptor blockers.
4. The "therapeutic window" concept for $P2Y_{12}$ receptor reactivity includes an upper threshold associated with ischemic events and a lower threshold associated with bleeding.
5. Large prospective trials utilizing the VerifyNow assay have thus far failed to demonstrate that personalized antiplatelet therapy is effective in reducing ischemic events; however, these trials have important limitations.
6. At this time, in the absence of strong prospective evidence to support personalized antiplatelet therapy, clinical decision-making about antiplatelet therapy rests on the large body of observational data and the fundamental importance of platelet physiology in catastrophic event occurrence in patients with high-risk coronary artery disease.

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Mouse Models of Thrombosis

Deya Cherpokova and Bernhard Nieswandt

Abstract

Platelet activation and aggregation at sites of vascular injury is essential to prevent excessive blood loss, but may also trigger life-threatening cardio- and cerebrovascular disease conditions. In vitro studies have significantly contributed to the current knowledge of central signaling pathways underlying platelet activation. However, in vitro experimental conditions cannot reproduce the complex environment with specific hemodynamic conditions in which interactions between platelets, other blood cell types, and the vessel wall take place. Intravital microscopy studies in experimental animal models, in particular mice, have advanced our understanding of the spatiotemporal characteristics and molecular pathways controlling thrombus formation in vivo. State-of-the-art imaging modalities together with essential tools allowing manipulations of the mouse genome have enabled us to explore new avenues toward the development of novel effective, yet safe antiplatelet drugs. This chapter summarizes widely used techniques to study in vivo thrombus formation and highlights data obtained in mouse models on the role of distinct platelet surface receptors, soluble platelet agonists, and the intrinsic coagulation cascade in thrombosis. Based on experimental evidence, the suitability of several platelet receptors and coagulation factors as novel targets for antithrombotic therapy is discussed.

Introduction

Platelets are small anucleate blood cells that survey the integrity of the vessel wall. They are constantly produced by their bone marrow-resident precursors, the megakaryocytes, to maintain the normal range of circulating platelet numbers (150–400 $\times$ 10^3/μl in humans and 1000 $\times$ 10^3/μl in mice). Most of the platelets never undergo adhesion before they are removed from the circulation by resident macrophages in the spleen and liver (Grozovsky et al. 2010). However, upon damage to the vessel wall, platelets come into contact with components of the exposed extracellular matrix which induces their activation, adhesion, and aggregation, followed by the formation of a thrombus that seals vessel lesions, minimizes blood loss, and prevents infections. Importantly, if uncontrolled thrombus formation occurs in pathological conditions, such as found in diseased atherosclerotic vessels, this may lead to life-threatening occlusive cardio- and cerebrovascular disease states which currently represent the leading cause of death and permanent disability worldwide (Lozano et al. 2012). Therefore, cellular activation needs to be tightly regulated by a complex interplay between activating and inhibitory mechanisms in platelets that ensure spatially and temporally controlled responses.

Currently available treatment options in acute ischemic disease states are limited, and antiplatelet agents are contraindicative in many cases because of increased incidence of bleeding complications that often outweigh therapeutic benefits. The adverse effects of clinically used platelet inhibitors on primary hemostasis emphasize the necessity to

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identify and characterize novel pharmacological targets for antiplatelet drugs with a powerful, yet safe antithrombotic profile. Recent advances in the development of novel therapeutic approaches have been greatly facilitated by studies in (genetically modified) mice that have significantly broadened our knowledge of the cellular and molecular interactions underlying hemostatic plug formation and pathological thrombosis. This chapter summarizes murine models that have substantially contributed to our understanding of in vivo arterial thrombus formation and may become instrumental for the development of both therapeutic strategies and new research directions (Table 1).

Mice as a Model Organism in Thrombosis Research

Considerable effort has been made to develop animal models for the investigation of platelet-dependent physiological and pathophysiological processes, such as hemostatic plug formation, arterial and venous thrombosis, atherosclerosis, and wound healing. During the past two decades, major improvements in intravital microscopy and the possibility to generate genetically engineered animal models have greatly facilitated investigations on the role of different platelet receptors and signaling cascades in in vivo thrombus formation, and have provided valuable insights into critical steps of this multifaceted process. Most of the key results summarized and discussed in this chapter have been obtained in mice which remain the most frequently used species in thrombosis research, particularly because of the following critical features: similarity to humans in anatomy, physiology and genetics, high fertility and short reproduction times, small size, and relatively low breeding costs (Sachs and Nieswandt 2007). Significantly, the invention of genetic methods that allow targeted manipulations of the mouse genome has been instrumental in unraveling the function of specific proteins in platelets. A major breakthrough was the generation of the platelet factor 4 (Pf4)-Cre transgenic mouse line that carries the Cre recombinase under the control of the Pf4 promoter and thus enables researchers to generate mouse lines with a megakaryocyte-/platelet-specific gene deletion (Tiedt et al. 2007). Of note, the recently developed CRISPR (clustered, regularly interspaced, short palindromic repeat)-Cas9 system is a highly efficient technology for genome editing of mouse zygotes (Wang et al. 2013; Sander and Joung 2014) that will undoubtedly further facilitate the generation of mutant mouse strains and will thereby also promote in vivo studies on both platelet production and on signaling pathways which control thrombus growth and resolution.

Although the mouse genome shares a substantial similarity with the human genome (approximately 95 %), thus emphasizing the suitability of the mouse as a model organism to study the role of numerous proteins in health and disease, direct extrapolation of data and translation of therapeutic approaches from the animal model to patient care may not be possible due to several differences between the two species (Sachs and Nieswandt 2007; Kim et al. 2010). Significant differences between mice and humans include the peripheral platelet count (150–400 $\times$ 10^3/μl in humans and 1000 $\times$ 10^3/μl in mice), the platelet life span (10 vs. 5 days), their diameter (3–4 μm vs. 1–2 μm), and, correspondingly, the platelet volume. Finally, the expression pattern of individual proteins is not identical between both species e.g., the alternative use of protease-activated receptors (PARs), the absence of the FcRγIIA receptor on mouse platelets. Nonetheless, these differences are significantly outweighed by the similarities and the suitability of laboratory mice for research purposes.

Importantly, inherent differences between inbred mouse strains with regard to platelet aggregation in vitro, hemostatic parameters, occlusive thrombus formation upon vascular injury, activity of tissue factor (TF), and TF pathway inhibitor (TFPI) have been increasingly recognized (Sudo et al. 2006; White et al. 2010). In addition, the role of environmental clues, housing, and husbandry have gained particular attention in recent years, and it has been recommended to use littermates and establish congenic colonies. Besides mice, rats, rabbits, zebrafish, guinea pigs, pigs, dogs, and nonhuman primates have been used in thrombosis research. Especially advanced phase investigations of novel thrombolytics have been predominantly performed in baboons due to the significant similarity to human (patho-)physiology. Nevertheless, because of ethical, experimental, methodological, and economic aspects, studies on in vivo thrombus formation will likely continue to use primarily murine models of thrombosis. In the following, we will provide a summary of the most commonly used methods for the induction of arterial thrombosis in mice and will subsequently focus on the role of central platelet adhesion receptors and downstream signaling pathways for initiation and stabilization of growing thrombi in vivo.

Platelet Activation and Thrombus Formation at Sites of Injury

At sites of vascular injury, subendothelial matrix components and several plasma constituents become immobilized onto exposed tissue. Thereby, different proteins, including von Willebrand factor (vWF), fibrillar collagens, fibronectin, and laminin, come into contact with circulating platelets. These interactions induce (1) initial platelet contact with the exposed

Table 1 Phenotype of mice with defined defects in platelet surface receptors discussed in this book chapter

Targeted protein	Impact on hemostasis[a]	References	Impact on arterial thrombosis	Type of injury	Reference
GPIbα (blockade)	Yes (moderate)	Kleinschnitz et al. (2007)	Yes, abolished thrombus formation	Ligation of the carotid artery	Massberg et al. (2003)
hIL-4Rα^{tg}/mGPIbα$^{-/-}$	Yes	Kanaji et al. (2002)	Yes, abolished platelet adhesion and aggregation	$FeCl_3$-injured mesenteric arterioles	Bergmeier et al. (2006)
				$FeCl_3$-injured carotid artery	Konstantinides et al. (2006)
Gp5$^{-/-}$	No (or shorter bleeding times)	Kahn et al. (1999), Ramakrishnan et al. (1999)	Yes, delayed formation of occlusive thrombi	$FeCl_3$-injured mesenteric arterioles	Moog et al. (2001)
			Yes, slightly accelerated thrombus formation, frequent embolization	$FeCl_3$-injured mesenteric arterioles	Ni et al. (2001)
GPVI depletion	Yes (mild to moderate)	Nieswandt et al. (2001c), Bender et al. (2013)	Yes, defective platelet adhesion and thrombus formation	Ligation or guidewire injury of the carotid artery	Massberg et al. (2003)
			Yes, defective thrombus formation	$FeCl_3$-injured mesenteric arterioles and carotid artery; mechanically injured abdominal aorta	Bender et al. (2011, 2013)
			Yes	Protection from collagen/epinephrine-induced thromboembolism	Nieswandt et al. (2001c)
			No	$FeCl_3$-injured carotid artery	Eckly et al. (2011)
Gp6$^{-/-}$	No or very mild	Kato et al. (2003), Lockyer et al. (2006), Bender et al. (2013)	Yes, increased recanalization	$FeCl_3$-injured carotid artery	Konstantinides et al. (2006)
			Yes, defective thrombus formation	$FeCl_3$-injured mesenteric arterioles and carotid artery; mechanically injured abdominal aorta	Bender et al. (2011)
Fcer1g$^{-/-}$	No	Mangin et al. (2006)	Yes, reduced platelet adhesion	Spring wire-injured femoral arteries	Konishi et al. (2002)
			Yes, defective platelet adhesion and thrombus formation	Ligation of the carotid artery	Gruner et al. (2005)
			Yes, defective thrombus formation	$FeCl_3$-injured mesenteric arterioles	Dubois et al. (2006)
			Yes, reduced thrombus formation	Moderate laser-induced injury of the mesenteric arterioles	Mangin et al. (2006)
			Yes	Protection from collagen/epinephrine-induced thromboembolism	Pozgajova et al. (2006)
			No	Laser-induced injury of the cremaster muscle arterioles	Dubois et al. (2006)
			No	"Folts-type" carotid artery thrombosis model, electrolytic injury of the carotid artery, severe laser-induced injury of the mesenteric arterioles	Mangin et al. (2006)
CLEC-2 depletion	Yes (moderate)	May et al. (2009), Bender et al. (2013)	Yes, defective thrombus formation	$FeCl_3$-injured mesenteric arterioles	May et al. (2009), Bender et al. (2013)
Clec-2$^{-/-}$ fetal liver cell chimeras	No	Suzuki-Inoue et al. (2010)	Yes, impaired thrombus formation	Laser-induced injury of the mesenteric arterioles	Suzuki-Inoue et al. (2010)
Clec-2$^{fl/fl,\ PF4}$	No or very mild	Bender et al. (2013)	Yes, delayed vessel occlusion	$FeCl_3$-injured mesenteric arterioles	Bender et al. (2013)
Itga2$^{-/-}$	No	Holtkotter et al. (2002), Chen et al. (2002)	Yes, mildly reduced thrombus stability	Photochemically induced injury of the carotid artery	He et al. (2003)
				$FeCl_3$-injured mesenteric arterioles	Kuijpers et al. (2007)
			No	Ligation of the carotid artery	Gruner et al. (2003)

(continued)

Table 1 (continued)

Targeted protein	Impact on hemostasis[a]	References	Impact on arterial thrombosis	Type of injury	Reference
Itgb1 $^{fl/fl, Mx\text{-}Cre}$	No	Nieswandt et al. (2001b)	No	Ligation of the carotid artery	Gruner et al. (2003)
				$FeCl_3$-injured carotid artery	Eckly et al. (2011)
Itgb3 $^{-/-}$	Yes	Hodivala-Dilke et al. (1999)	Yes, abolished thrombus formation	$FeCl_3$-injured carotid artery	Smyth et al. (2001)
Itga2b $^{-/-}$	Yes	Tronik-Le Roux et al. (2000)	Yes, abolished thrombus formation	$FeCl_3$-injured carotid artery	Eckly et al. (2011)
P2ry1 $^{-/-}$	Yes (moderate)	Fabre et al. (1999), Leon et al. (1999)	Yes	Protection from collagen-/epinephrine- and collagen-/ADP-induced thromboembolism	Fabre et al. (1999), Leon et al. (1999)
P2ry12 $^{-/-}$	Yes	Foster et al. (2001), Andre et al. (2003)	Yes, abolished thrombus formation	$FeCl_3$-injured mesenteric arterioles	Andre et al. (2003)
Par3 $^{-/-}$	Yes (moderate)	Weiss et al. (2002)	Yes, impaired thrombus formation	$FeCl_3$-injured mesenteric arterioles	Weiss et al. (2002)
Par4 $^{-/-}$	Yes	Sambrano et al. (2001), Hamilton et al. (2004)	Yes, reduced occlusive thrombus formation	$FeCl_3$-injured mesenteric arterioles	Sambrano et al. (2001)
			Yes, reduced platelet accumulation	Laser-induced injury of the cremaster muscle arterioles	Vandendries et al. (2007)
Tp $^{-/-}$	Yes	Thomas et al. (1998)			
Adra2a $^{-/-}$	Yes (moderate)	Pozgajova et al. (2006)	Yes, compromised thrombus stability and frequent embolization	$FeCl_3$-injured mesenteric arterioles; mechanically injured abdominal aorta	Pozgajova et al. (2006)
			Yes	Protection from collagen-/epinephrine-induced thromboembolism	Pozgajova et al. (2006)

[a]As assessed by tail bleeding time assays

extracellular matrix (ECM) (tethering); (2) adhesion, activation, and release of second-wave mediators to reinforce persistent activation; and (3) firm adhesion to the ECM and thrombus growth (Fig. 1).

Initial platelet tethering to exposed components of the ECM is mediated by binding of immobilized vWF to its major ligand on the platelet surface—glycoprotein (GP) Ibα (Savage et al. 1998). GPIbα-vWF interactions result in rapid deceleration of circulating platelets, but do not lead to firm adhesion to the ECM. Instead, platelet rolling enables binding of collagen to its major activating receptor on the platelet surface—GPVI (Nieswandt and Watson 2003). Collagen-activated platelets undergo a series of signaling events, culminating in a sustained rise of intracellular Ca^{2+} concentration, surface exposure of negatively charged procoagulant phosphatidylserine (PS), rearrangements of the cytoskeleton, mobilization of α- and dense granules, and release of soluble platelet agonists, such as adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2) (Nieswandt et al. 2011; Dutting et al. 2012). Activated platelets also release polyphosphates that are considered to potently activate coagulation factor XII (FXII, Hageman factor)—the starting point of the intrinsic coagulation cascade (Muller et al. 2009). Furthermore, PS on the outer leaflet of the platelet membrane provides high-affinity binding sites for coagulation factors and together with exposed TF triggers thrombin generation (Heemskerk et al. 2013). Locally generated thrombins, ADP and TxA_2 reinforce cellular activation in an autocrine and paracrine fashion and recruit additional platelets to the growing thrombus. Finally, extra- and intracellular signaling events culminate in the induction of a conformational change of integrin adhesion receptors, most notably $\alpha_{II}b\beta_3$ (GPIIb/IIIa), from a low- to high-affinity state which triggers firm platelet adhesion and aggregation, and stabilizes the growing thrombus (Varga-Szabo et al. 2008).

Mouse Models for Assessment of Hemostatic Functions

An important requirement for antithrombotic agents is that they prevent thrombosis while only mildly affecting hemostatic functions. A major side effect of several widely used antiplatelet drugs is their adverse effect on primary hemostasis and the increased incidence of bleeding complications. The overall hemostatic function in mice is most frequently assessed in the tail bleeding assay, in which a small segment of the tail tip is cut by a surgical blade and the time to cessation of bleeding recorded (Fig. 2). Besides measurement of bleeding times, further important parameters include determination of total blood volume loss, rebleeding, and animal survival after challenge. Upon removal of the tail tip,

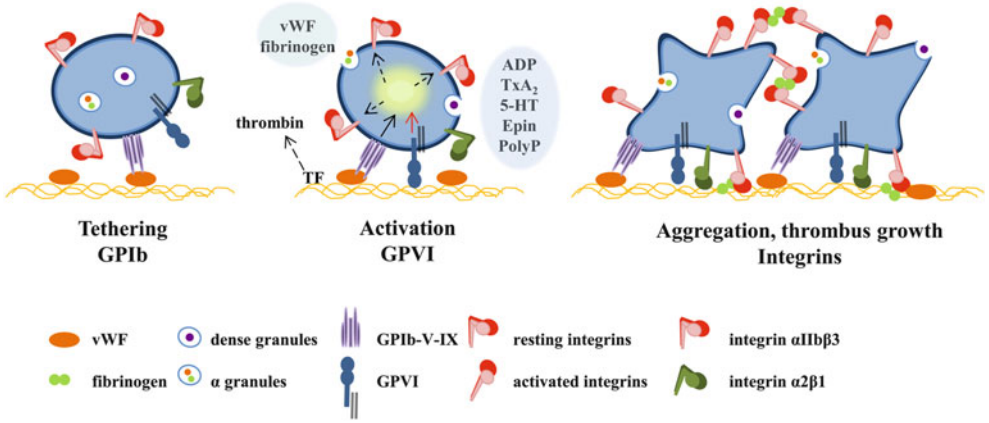

Fig. 1 Simplified model of thrombus formation. At sites of vascular injury, initial contact (tethering) of platelets with immobilized components of the exposed ECM is enabled through GPIb-vWF interactions. Subsequently, GPVI can bind to collagen which initiates a powerful signaling cascade that mediates increase in cytosolic Ca^{2+} concentration (not shown), release of α- and dense granules, and a conformational change of integrins to a high-affinity state. Released platelet agonists, such as ADP and thromboxane A_2 (TxA_2), together with locally produced thrombin, reinforce cellular activation in an autocrine and paracrine fashion. Activated integrins support firm platelet adhesion to the ECM which together with locally secreted or synthesized platelet agonists facilitates thrombus growth. *TF* tissue factor, *Epin*. epinephrine

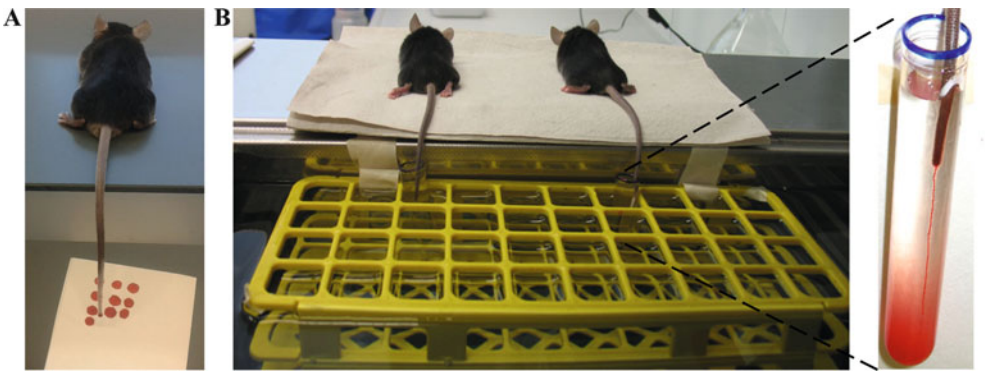

Fig. 2 Mouse models for assessment of hemostatic functions. (**a**) The tail tip of an anesthetized mouse is cut using surgical blade, and blood is gently adsorbed on filter paper without making contact with the wound site at 20-s intervals until cessation of blood loss. (**b**) Clipped mouse tail tips are immersed in warm (37 °C) 0.9 % isotonic saline

the tail is immersed in warm saline or PBS or blood is collected with Whatman paper or in a reaction tube.

Notably, the tail bleeding assay is susceptible to considerable variability even within the same mouse strain, and multiple factors may influence the outcome. For instance, the genetic background accounts for variations in total blood loss between different mouse strains (Schiviz et al. 2014). In addition, gender, age, weight, and, importantly, the type of anesthesia play a critical role for the time of cessation of bleeding. Of note, increased tail bleeding times do not necessarily correlate with compromised ability of platelets to maintain the vascular integrity at sites of thrombosis and inflammation. For example, blockade of the vWF receptor on the platelet surface—GPIbα—significantly prolongs the time to cessation of blood loss in the tail tip model, but leads to a profound protection in a murine model of acute ischemic stroke, without increasing the incidence of intracranial hemorrhage (Kleinschnitz et al. 2007) (Stoll et al 2017 in this volume). In summary, despite its dependence on different variables, the tail tip assay remains the most frequently used test of hemostatic functions in mice.

Murine Models of In Vivo Arterial Thrombus Formation

Although a substantial body of knowledge about the central signaling pathways mediating platelet activation has been gained by in vitro studies, a major advance in our understanding of the complex interplay between platelets, the vessel wall, and local hemodynamic conditions that govern thrombus growth has been achieved only after the introduction of

intravital microscopy techniques. Widely used in vivo arterial thrombus formation models differ in the size of the vascular bed, and the method to induce endothelial denudation by laser, chemical, or mechanical injury.

One largely used technique to study thrombus formation in the microcirculation by fluorescence intravital microscopy is the laser-induced vessel wall injury model (Falati et al. 2002; Rosen et al. 2001). The intensity of the laser power in this minimally invasive injury model can be varied to modulate the severity of injury which in turn has been shown to determine the major pathways governing platelet activation at the site of injury (Nonne et al. 2005). The exposure of collagen fibers has been proposed to be the predominant mechanism underlying thrombus formation upon superficial injury, whereas a more severe injury leads to massive thrombin generation which appears to be sufficient to drive thrombus formation largely independently of collagen-induced activation events (Mangin et al. 2006). Of note, laser-induced endothelial denudation leads to rapid platelet accumulation at sites of injury which, however, typically triggers nonocclusive thrombus formation.

Recent seminal work has provided insight into the complex architecture of a hemostatic plug in the microcirculation formed after laser injury. These experiments revealed that platelet activation is not necessarily uniformly distributed, but is rather heterogeneous and depends on the spatial and temporal regulation of agonist distribution within the thrombus (Stalker et al. 2014). Based on the expression of α-granule P-selectin on the platelet surface upon powerful cellular activation, observational studies detected the presence of a stable densely packed core close to the injury site composed of fully activated platelets and a more loosely organized and accordingly less stable outer shell built by P-selectin-negative platelets (Stalker et al. 2013). Detailed investigations also identified a distinct distribution of soluble platelet agonist gradients. Thrombin generation and fibrin deposition are typically found in the core region, adjacent to the laser-induced lesion, and are spatially restricted to the distribution of procoagulant membranes of platelets and endothelial cells at the site of injury (Stalker et al. 2013; Ivanciu et al. 2014; Welsh et al. 2014). By contrast, inhibition of the activity of ADP, which is secreted from platelet dense granules or released from damaged cells at injury sites, plays an important role for the reduction of the outer shell region (Stalker et al. 2013). The localization of TxA_2, which is generated and secreted by activated platelets and reinforces cellular activation in an autocrine and paracrine fashion, is less well defined, and likely depends predominantly on its major source—activated platelets (Stalker et al. 2014). Recent computational models ascribed the different distribution of soluble agonists to the physically heterogeneous structure and density of the platelet plug that accounts for distinct transport properties of the core and the shell region (Welsh et al. 2014; Stalker et al. 2014). Taken together, a better understanding of the complex architecture of the thrombus with a defined localization of prominent platelet agonists may have important implications for both the use of currently available therapies and the development of new antithrombotics.

Chemical injury by a topical application of ferric chloride ($FeCl_3$) onto the surface of a vessel is one of the most frequently used experimental procedures to induce thrombosis in rodents (Fig. 3) (Schoenwaelder and Jackson 2015). The method was initially described for the induction of arterial thrombosis in rats (Kurz et al. 1990) and is meanwhile one of the most commonly applied techniques to investigate in vivo thrombus formation in mice because of the relative simplicity of implementation. Ferric chloride is typically applied to vessels accessible to a Doppler flow probe (such as the common carotid artery) to monitor the blood flow rate upon injury. Alternatively, accumulation of labeled platelets and the subsequent formation of an occlusive thrombus at sites of injury can be visualized by intravital microscopy of arterioles in the mesentery or the cremaster muscle.

Despite its common use, the exact mechanisms of $FeCl_3$-induced thrombosis are not fully elucidated yet, and therefore remain controversially discussed. A long-standing theory that the major effects of this type of chemical injury are restricted to the vasculature has been recently refined. A few studies have demonstrated that ferric chloride is taken up by endothelial cells and afterwards released into the vessel lumen (Tseng et al. 2006; Eckly et al. 2011). Several investigators then assumed that the high concentration of iron and potentially reactive oxygen species generation may lead to endothelial denudation and subsequent exposure of subendothelial components that trigger thrombosis (Kurz et al. 1990; Li et al. 2013). Importantly, however, further studies identified only minor endothelial lesions and collagen exposure and detected instead considerable accumulation of red blood cells at sites of $FeCl_3$-injury, followed by rapid platelet recruitment (Eckly et al. 2011; Barr et al. 2013; Woollard et al. 2009). Furthermore, TF was detected on ferric chloride particles which can mediate platelet adhesion and fibrin deposition (Eckly et al. 2011). Of note, the effects of $FeCl_3$ on the vasculature and on different blood cell populations likely depend on the applied concentration and exposure time, and differences in these two parameters may explain the controversy regarding the mode of action.

Another way to induce chemical injury that triggers arterial thrombosis is intravenous administration of the photoactive stain rose bengal. This substance rapidly accumulates in the membranes of endothelial and other cells upon application. A photochemical reaction is mediated by the exposure of an artery to green light (540 nm) which induces the generation of reactive oxygen species and lesion of the endothelium that fuels arterial thrombosis (Matsuno et al. 1991; Kikuchi et al. 1998; Westrick et al. 2007).

Finally, arterial thrombosis can be triggered by mechanical injury of large arteries (e.g., the abdominal aorta (Fig. 3),

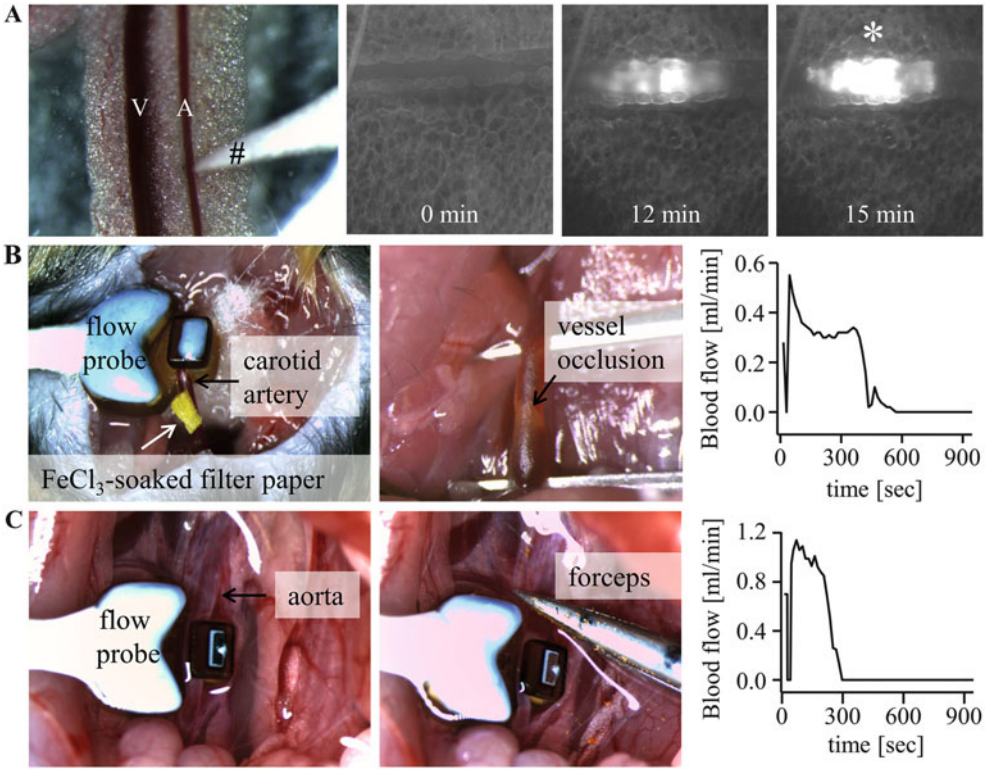

Fig. 3 Arterial thrombosis models. (**a**) Thrombus formation in $FeCl_3$-injured mesenteric arterioles. (*Left*) $FeCl_3$ is topically applied to an arteriole (**A**). *V* vein, *number sign* tip of the $FeCl_3$-soaked filter paper. (*Middle* and *right*) Accumulation of fluorescently labeled platelets at the site of injury at the indicated time points after $FeCl_3$-application as observed by intravital microscopy. *Asterisk* denotes stable vessel occlusion. (**b**) Thrombus formation in the $FeCl_3$-injured carotid artery. (*Left*) Experimental setup. Blood flow is monitored with an ultrasonic flow probe. (*Middle*) Stable vessel occlusion due to thrombus formation (*arrow*). (*Right*) Representative blood flow curve. (**c**) Mechanical injury of the abdominal aorta is triggered by a single firm compression with forceps (*middle*). (*Right*) Representative blood flow rates as measured with an ultrasonic flow probe

the common carotid arteries, or femoral arteries). Endothelial damage can be mediated indirectly by a firm compression of the vessel with a forceps or by ligation with a filament (Massberg et al. 2003; Renne et al. 2005), by combination of both techniques (Mangin et al. 2006), or directly by introducing a guidewire into the vessel (Konishi et al. 2002; Massberg et al. 2003). Thrombus formation following compression or ligation of arterial vessels occurs mainly through collagen-dependent mechanisms, as demonstrated by the profound protection from arterial thrombosis in mice deficient for GPVI, the central activating collagen receptor on the platelet surface (Massberg et al. 2003; Bender et al. 2011).

A relatively simple method to study thrombotic activity in mice is the infusion of a mixture of collagen and epinephrine or collagen and ADP which induces rapid platelet consumption due to intravascular thrombus formation and typically leads to lethal pulmonary thromboembolism in wild-type animals within a few minutes (DiMinno and Silver 1983). Radiolabeling of platelets (e.g., with ^{111}Indium oxine (Thakur et al. 1976)) can be applied for in vivo monitoring of aggregate formation in the lung after induction of thromboembolism (Oyekan and Botting 1986; Tymvios et al. 2008). Antibody-mediated depletion of GPVI (Nieswandt et al. 2001c) or its genetic deficiency (Pozgajova et al. 2006) provided a profound protection from collagen/epinephrine-induced thromboembolism. Likewise, mice lacking the α_{2A}-adrenergic receptor were resistant to thromboembolism in this model (Pozgajova et al. 2006), as were animals deficient for the ADP receptor $P2Y_1$ (Fabre et al. 1999; Leon et al. 1999) or mice treated with the selective $P2Y_1$ receptor antagonist MRS2500 (Hechler et al. 2006). Similarly, mice deficient for G-protein α-subunits, such as $G\alpha_q^{-/-}$ (Offermanns et al. 1997) or $G\alpha_z^{-/-}$ (Yang et al. 2000), were protected from the systemic effects triggered by the collagen/epinephrine challenge.

Alternatively, mechanisms of pulmonary embolism can be studied in a thrombin-dependent model in which infusion of thromboplastin (TF) mimics TF exposure (Leon et al. 2001). The thrombin receptors on mouse platelets PAR3 and PAR4 (Weiss et al. 2002; Hamilton et al. 2004) as well as the ADP receptor $P2Y_1$ have been identified to critically contribute to pulmonary thrombosis in this model.

The arterial thrombosis models presented here differ in the severity and extent of injury, vessel size, degree of vessel occlusion, and signaling pathways that initiate thrombus formation. Therefore, critical requirements to future research include a better understanding of the molecular mechanisms which predominantly drive thrombus growth in different injury models, increased recognition of advantages and disadvantages of each thrombosis model, and a recommendation to apply different models before drawing a conclusion on the role of distinct molecules in arterial thrombosis. Still, one major limitation that applies to all thrombosis models is the fact that mice are naturally not susceptible to atherothrombosis, and vessel occlusion is

induced in the healthy vasculature of experimental animals, whereas arterial thrombosis in humans occurs in diseased vessels.

Platelet Adhesion and Activation Receptors

GPIb

GPIb is part of the megakaryocyte-/platelet-specific GPIb-V-IX receptor complex and plays a crucial role for platelet tethering at sites of vascular injury through binding to vWF. Besides vWF, GPIb also interacts with P-selectin, thrombospondin, thrombin, macrophage antigen 1 (Mac-1), the coagulation factors XI and XII, and high-molecular-weight kininogen. Lack or dysfunction of GPIb-V-IX in humans is associated with the Bernard-Soulier syndrome (BSS), a rare inherited bleeding disorder characterized by the presence of giant platelets, thrombocytopenia and a bleeding phenotype (Andrews and Berndt 2013). These key features of the disease are recapitulated in mouse models deficient in GPIbα (Ware et al. 2000) or GPIbβ (Kato et al. 2004). Of note, mutations within genes encoding GPIbα, GPIbβ, and GPIX (but not GPV) have been identified to cause BSS, and, similarly, GPV-deficient mice do not reproduce the phenotype of the disease (Kahn et al. 1999).

The role of the GPIb-vWF axis for arterial thrombus formation has been extensively studied. Blockade of the vWF binding site on GPIbα by administration of anti-GPIbα Fab fragments (p0p/B) to wild-type mice significantly reduced platelet tethering to the injured carotid artery and correspondingly resulted in an abolished aggregate formation at sites of vascular injury (Massberg et al. 2003). Likewise, transgenic mice in which the extracellular domain of GPIbα was replaced by the α-subunit of the human IL-4 receptor exhibited a severe bleeding phenotype and were profoundly protected from $FeCl_3$-induced arterial thrombosis (Kanaji et al. 2002; Bergmeier et al. 2006; Konstantinides et al. 2006). Similarly, vWF-deficient mice displayed increased tail bleeding times or were unable to stop bleeding, and approximately 10 % of neonates were affected by spontaneous intra-abdominal bleeding (Denis et al. 1998). $vWf^{-/-}$ mice did not form occlusive thrombi in mesenteric arterioles upon ferric chloride-induced injury (Denis et al. 1998). Interestingly, however, platelet tethering was still occasionally observed in injured arterioles of $vWf^{-/-}$ mice and subsequently led to delayed appearance of first thrombi (Ni et al. 2000), demonstrating that the antithrombotic effects of GPIbα-deletion are more profound than those observed in mice deficient for its major ligand—vWF (Bergmeier et al. 2006; Denis et al. 1998). Importantly, the antithrombotic potential of targeting the GPIb-vWF axis has been documented also in baboons (Cauwenberghs et al. 2000; Wu et al. 2002).

Ultra-large vWF is a multimeric protein (up to 20,000 kDa) which is synthesized in endothelial cells and megakaryocytes. vWF multimers are rapidly cleaved in the plasma to less thrombogenic forms by the enzyme A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13 (ADAMTS13). The naturally occurring antithrombotic activity of ADAMTS13 is critical for dampening thrombosis, as revealed by accelerated thrombus formation in chemically injured arterioles of $Adamts13^{-/-}$ mice (Chauhan et al. 2006; Banno et al. 2009). Remarkably, concomitant deficiency of vWF abolishes the prothrombotic state in $Adamts13^{-/-}$ mice (Chauhan et al. 2008). Recombinant human ADAMTS13 which is currently in a phase I clinical study for the treatment of hereditary thrombotic thrombocytopenia purpura (TTP) (ClinicalTrails.gov identifier: NCT02216084) may have both antithrombotic and thrombolytic properties, but this awaits further experimental and clinical confirmation. An alternative approach to interfere with the GPIb-vWF axis could be the targeted downregulation of GPIbα from the platelet surface by proteolytical cleavage via A disintegrin and metalloproteinase 17 (ADAM17)/tumor necrosis factor-alpha-converting enzyme (TACE) (Bergmeier et al. 2004; Bender et al. 2010).

Further experimental support that interference with GPIb-vWF may become a potential approach for future treatment strategies in occlusive thrombotic disease conditions came from studies in mice which lack phospholipase D1 (PLD1). PLD1 activity is important for signaling transduction downstream of vWF-engaged GPIbα and thus controls GPIb-dependent $\alpha_{IIb}\beta_3$ activation and aggregate formation under high shear conditions (Elvers et al. 2010). Correspondingly, $Pld1^{-/-}$ mice were protected from arterial thrombosis in the chemically injured carotid artery and in the mechanically injured abdominal aorta, importantly, without affecting hemostasis (Elvers et al. 2010). In agreement with these findings, pharmacological blockade of PLD isoforms by the small molecule PLD inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide (FIPI), reproduced the protection from arterial thrombosis in wild-type mice, thereby demonstrating that modulating the signaling cascade downstream of GPIb-vWF may emerge as a novel therapeutic approach (Stegner et al. 2013).

The (Hem)ITAM Receptors GPVI and CLEC-2

The central activating collagen receptor GPVI is a megakaryocyte-/platelet-specific transmembrane type I receptor which is non-covalently associated with the FcRγ-chain that serves as the signaling subunit of the receptor complex

because it contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail (Nieswandt and Watson 2003). To date, a few patients with GPVI-associated defects have been reported. These defects have been ascribed to autoantibody-triggered receptor loss (Sugiyama et al. 1987; Boylan et al. 2004; Arthur et al. 2007) and compound heterozygous (Dumont et al. 2009; Hermans et al. 2009) and homozygous mutations in the *GP6* gene (Matus et al. 2013). Platelets isolated from these patients displayed abolished aggregation following stimulation with collagen, whereas the patients showed only a mild bleeding tendency. Likewise, genetic (Kato et al. 2003; Mangin et al. 2006; Lockyer et al. 2006; Bender et al. 2013) or antibody-induced deficiency of GPVI (Nieswandt et al. 2001c; Bender et al. 2013) had no or only a mild effect on tail bleeding times.

A large body of experimental data has documented a profound protection from arterial thrombosis induced in different vascular beds and by a variety of injury methods in wild-type mice depleted of GPVI (Massberg et al. 2003; Bender et al. 2011, 2013), in $Gp6^{-/-}$ mice (Konstantinides et al. 2006; Bender et al. 2011), or in FcRγ-deficient mice which also lack GPVI (Nieswandt et al. 2000; Konishi et al. 2002; Gruner et al. 2005; Dubois et al. 2006). By contrast, increased receptor density and enhanced GPVI signaling are associated with accelerated occlusive thrombus formation in mice (Cherpokova et al. 2015). Furthermore, the efficacy of GPVI inhibition as a promising novel antithrombotic strategy was further emphasized by studies in rats and nonhuman primates treated with Fab fragments of anti-GPVI antibodies (Matsumoto et al. 2006; Li et al. 2007; Ohlmann et al. 2008). Of note, a few studies identified GPVI-independent mechanisms of arterial thrombus formation which are likely related to altered severity or extent of vascular injury (Mangin et al. 2006; Dubois et al. 2006; Eckly et al. 2011). Remarkably, previously unrecognized properties of GPVI to bind to polymerized fibrin have been only very recently identified, and these interactions may have an important function for the stabilization of the growing thrombus (Mammadova-Bach et al. 2015; Alshehri et al. 2015).

Based on the findings that GPVI deficiency, blockade, or antibody-mediated depletion in mice and other experimental animals provides powerful protection from arterial thrombosis without affecting hemostasis, the receptor has emerged as an attractive potential target for antithrombotic therapy (Dutting et al. 2012). Current GPVI targeting strategies under development focus on three different approaches. They include (1) blockade of the GPVI-collagen interaction, (2) immunodepletion of GPVI via monoclonal antibodies, or (3) interference with key signaling molecules downstream of the receptor, such as the tyrosine kinase Syk (Stegner et al. 2014). GPVI immunodepletion by monoclonal antibodies induces specific and irreversible removal of the receptor from the surface of murine platelets, resulting in a GPVI-knockout-like phenotype for at least 2 weeks which is accompanied only by a short thrombocytopenia and a transiently reduced PAR4 activity at early time points after antibody administration (Nieswandt et al. 2001c; Schulte et al. 2003, 2006). Antibody-triggered GPVI downregulation in vivo is still incompletely understood, but experimental evidence suggests that receptor depletion occurs through two principal mechanisms: ectodomain shedding and internalization followed by intracellular degradation (Nieswandt et al. 2001c; Boylan et al. 2006; Rabie et al. 2007; Takayama et al. 2008; Bender et al. 2010). Both mechanisms require signaling through the ITAMs in the FcRγ-chain (Rabie et al. 2007). Importantly, studies in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice demonstrated that GPVI can also be removed from the surface of circulating human platelets (Boylan et al. 2006). Similar to the effects observed in mice following antibody-induced GPVI downregulation (Schulte et al. 2006), immunodepletion of GPVI from the surface of human platelets was associated with reduced responsiveness to G-protein-coupled receptor (GPCR) activation, implicating that removal of GPVI may have a broader impact on platelet function than previously assumed (Boylan et al. 2006).

Although compelling experimental data corroborate the notion that GPVI may become a promising antithrombotic target, careful assessment of the safety profile of an anti-GPVI therapy is warranted. GPVI immunodepletion severely compromises the overall hemostatic capacity in mice deficient for the hemITAM receptor C-type lectin-like receptor 2 (CLEC-2) (Boulaftali et al. 2013; Bender et al. 2013), another collagen receptor on the platelet surface—α2β1, or concomitantly treated with acetylsalicyclic acid (ASA) (Gruner et al. 2004). These findings reveal a critical functional redundancy between (hem)ITAM-bearing receptors, on the one hand, and between collagen receptors, on the other, in the maintenance of platelet hemostatic functions in mice which may explain the mild bleeding tendency in the absence of GPVI in mice and humans. Moreover, these experimental data emphasize the necessity to evaluate the functional status of other receptors prior to and during GPVI targeting.

Based on initial studies on the role of the second (hem) ITAM-containing receptor on mouse platelets CLEC-2 in thrombosis and hemostasis, it has been proposed that the receptor might become a target for antithrombotic therapy. Antibody-mediated downregulation of CLEC-2 from circulating platelets (May et al. 2009) or genetic ablation of the receptor (Suzuki-Inoue et al. 2010) was accompanied by a significant protection from occlusive arterial thrombosis in mice and resulted only in a moderate increase in bleeding

times. However, recent findings that revealed a pivotal role for CLEC-2 in a plethora of processes, including development of lymph nodes, maintenance of the integrity of high endothelial venules, and blood/lymph vessel separation, question the suitability of the hemITAM receptor as a safe antithrombotic target (Boulaftali et al. 2014). The third ITAM-bearing receptor—FcγRIIa—is not expressed on the surface of mouse platelets. A study utilizing a transgenic mouse model that expresses the human FcγRIIa (McKenzie et al. 1999) demonstrated enhanced arterial thrombus formation in the laser-injured cremaster muscle arterioles (Zhi et al. 2013). These data together with the well-established role of FcγRIIa in the pathogenesis of several disease conditions related to immune-mediated thrombocytopenia and thrombosis syndrome suggest that modulating the activity of the receptor may have important implications in future antithrombotic and anti-inflammatory therapies.

A naturally occurring mechanism to attenuate constitutive signaling through (hem)ITAM receptors is mediated by the action of immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing receptors. To date, the expression of a few ITIM-containing receptors in platelets has been described, including platelet endothelial cell adhesion molecule-1 (PECAM-1), G6b-B, TREM-like transcript-1 (TLT-1), and carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM1) and CEACAM2. Deficiency of PECAM-1 (Falati et al. 2006), CEACAM1 (Wong et al. 2009), or CEACAM2 (Alshahrani et al. 2014) was associated with slightly enhanced arterial thrombus formation upon injury which demonstrates a minor inhibitory role of individual ITIM receptors in this process and points toward a substantial functional redundancy among inhibitory mechanisms in platelets. In addition, a few proteins which interact with the GPVI signalosome, such as CLP36 (Gupta et al. 2012) and Src-like adapter proteins (SLAP/SLAP2) (Cherpokova et al. 2015), have been identified, and deficiency of these molecules resulted in a profound prothrombotic phenotype, thus revealing an important inhibitory function of the adapter molecules in dampening excessive ITAM signaling at sites of vascular injury.

α2β1

The second prominent collagen receptor on the platelet surface—integrin α2β1—contributes to, but is not essential for, platelet adhesion on collagen under flow (Nieswandt et al. 2001b; Nieswandt and Watson 2003). Antibody-induced blockade of α2 or genetic deficiency of the integrin subunit in mice (*Itga2*$^{-/-}$) was reported to have no or only a very minor inhibitory effect on arterial thrombus formation (Holtkotter et al. 2002; Chen et al. 2002; He et al. 2003; Gruner et al. 2003; Kuijpers et al. 2007; Shida et al. 2014). These rather mild effects could be attributed to the still largely intact response of the platelets to collagen via GPVI and platelet adhesion to multiple components of the ECM through other integrins (most notably $\alpha_{IIb}\beta_3$). In line with the findings in α2-deficient mice, deletion of integrin β1 specifically in platelets, which besides α2β1, also ablates α5β1 (fibronectin receptor) and α6β1 (laminin receptor) expression, did not affect platelet aggregation and stable thrombus formation in the injured carotid artery (Gruner et al. 2003). Furthermore, these studies also pointed toward a principal role of integrin $\alpha_{IIb}\beta_3$ for firm platelet adhesion at sites of vascular injury (Gruner et al. 2003).

Platelet Aggregation

As mentioned above, platelet aggregation plays a pivotal role for the formation of a stable thrombus at the injured vessel wall. Key to this process is the activity of the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). Integrin $\alpha_{IIb}\beta_3$ supports thrombus stabilization through binding to fibrinogen, thereby mediating bridging of adjacent cells. In addition, $\alpha_{IIb}\beta_3$ interacts with several other proteins exposed on the ECM, including fibronectin, vWF, thrombospondin, and vitronectin (Bergmeier and Hynes 2012). Congenital deficiency or dysfunction of integrin $\alpha_{IIb}\beta_3$ in humans is associated with Glanzmann thrombasthenia characterized by a severe bleeding diathesis and inability of platelets to adhere (Nurden et al. 2013). Similarly, mice deficient for either αIIb (GPIIb) or β3 (GPIIIa) integrin subunit phenocopied key features of the disease by displaying defective platelet aggregation, clot retraction and severe hemostatic defects and, in addition, abolished thrombus formation in injured vessels (Hodivala-Dilke et al. 1999; Tronik-Le Roux et al. 2000; Smyth et al. 2001). Likewise, mice lacking the major integrin $\alpha_{IIb}\beta_3$ ligand—fibrinogen—suffer from severe bleedings after birth, and their platelets failed to aggregate or induce blood clotting (Suh et al. 1995). Mice which express a truncated form of fibrinogen (FgγΔ5) that lacks the integrin $\alpha_{IIb}\beta_3$ binding motif, but conversion to fibrin remains unaffected by the mutation, could not stop bleeding from the tail tip, but were able to prevent excessive blood loss at other injury sites and displayed unaltered clot retraction in vitro (Holmback et al. 1996). Interestingly, both fibrinogen-deficient and FgγΔ5 mice formed thrombi in $FeCl_3$-injured mesenteric arterioles which, however, often embolized, thereby emphasizing the critical role of fibrinogen for thrombus stabilization by bridging $\alpha_{IIb}\beta_3$ of adjacent platelets (Ni et al. 2000, 2003a). Unexpectedly, occlusive thrombus formation still occurred in mice with a fibrinogen/vWF double deficiency, albeit thrombi were more fragile, leading to frequent embolization and resultant vessel occlusion downstream of the site of injury (Ni et al. 2000). Recent

studies identified that fibronectin, which is required for stable thrombus growth in $FeCl_3$-injured arterioles (Ni et al. 2003b), likely accounts for fibrinogen/vWF-independent platelet aggregation and occlusive thrombus formation (Wang et al. 2014).

To date, a plethora of proteins have been identified to interact with cytoplasmic tails of integrin subunits and have been proposed to modulate integrin inside-out and outside-in signaling (Nieswandt et al. 2009). However, the exact contribution of numerous of these proteins to integrin activation is still under investigation or has been controversially discussed. By contrast, the functional significance of only a few of these molecules has been elucidated in detail. For instance, talin-1 which binds to the cytoplasmic tail of both αIIb and β3 is essential for integrin activation, platelet aggregation, platelet hemostatic functions, and stable thrombus formation in injured vessels (Nieswandt et al. 2007; Petrich et al. 2007). Likewise, deficiency of another critical component of the integrin activation machinery in platelets—kindlin-3—resulted in abrogated activation of $\alpha_{IIb}\beta_3$ and β1 integrins and was accompanied by a severe hemostatic defect and abolished arterial thrombus formation (Moser et al. 2008). Importantly, mutations in *FERMT3*, the gene encoding human kindlin-3, have been identified in humans suffering from a disease condition termed leukocyte adhesion deficiency III (LAD-III), characterized by a severe bleeding diathesis, dramatically increased susceptibility to infections and poor wound healing, among other symptoms (Malinin et al. 2009; Mory et al. 2008; Svensson et al. 2009).

Several molecules have been implicated to be critically involved in orchestrating the complex signaling cascade that eventually leads to talin-1/kindlin-3-dependent integrin activation. Among the numerous constituents of this signaling machinery, Rap1, Ca^{2+} and diacylglycerol-regulated guanine-nucleotide-exchange factor 1 (CalDAG-GEFI) and RASA3, but unexpectedly not Rap1-GTP-interacting adapter molecule (RIAM), have been demonstrated to control hemostasis and arterial thrombosis in mice (Chrzanowska-Wodnicka et al. 2005; Crittenden et al. 2004; Stolla et al. 2011; Stefanini et al. 2015; Stritt et al. 2015).

Soluble Platelet Agonists

Thrombin

Thrombin is rapidly generated at sites of vascular injury and induces rapid and powerful platelet activation via receptors of the PAR family (PAR1 and PAR4 in human platelets and PAR3 and PAR4 in mouse platelets). Absence of PAR3 or PAR4 is associated with a profound protection in murine models of arterial thrombosis (Kahn et al. 1998; Weiss et al. 2002; Sambrano et al. 2001; Vandendries et al. 2007). Interestingly, PAR3-deficient platelets displayed a weak response to thrombin, whereas PAR4-deficiency was accompanied by a complete resistance to thrombin activation, emphasizing that PAR4 is the sole signaling protease-activated receptor for thrombin in mouse platelets (Weiss et al. 2002; Sambrano et al. 2001). A series of intravital microscopy studies revealed that the activity of thrombin is spatially and temporally restricted to the stable core of the growing thrombus composed of fully activated platelets where thrombin plays a critical role for its stabilization (Stalker et al. 2013, 2014).

Other Platelet Agonist Operating via GPCRs

ADP

ADP is released from platelet dense granules and other activated cells at the site of injury and potentiates platelets responses by engagement of two receptors—$P2Y_1$ and $P2Y_{12}$. $P2Y_1$-deficient mice displayed moderately increased tail bleeding times and a significant protection in a model of collagen/epinephrine-induced thromboembolism (Fabre et al. 1999; Leon et al. 1999). The significance of $P2Y_{12}$ for platelet activation is exemplified by currently available $P2Y_{12}$ antagonists that efficiently inhibit platelet activation and protect from thrombotic events in humans (Michelson 2010). This hallmark of $P2Y_{12}$ blockade is recapitulated in mice which were treated with $P2Y_{12}$ antagonists or genetically engineered mice that lack $P2Y_{12}$ or $G\alpha_{i2}$, the principal G-protein coupled to $P2Y_{12}$, which displayed unstable nonocclusive arterial thrombus formation, accompanied, however, by prolonged tail bleeding times (Foster et al. 2001; Andre et al. 2003; Stolla et al. 2011; Devanathan et al. 2015). Recent elegant studies demonstrated that $P2Y_{12}$ inhibition is most efficient in reducing the size of the outer shell of loosely packed platelets, whereas $P2Y_{12}$ antagonists did not significantly affect the thrombus core (Stalker et al. 2013). These findings provide an important insight into the mode of action of clinically used $P2Y_{12}$ antagonists and help to explain their safety profile.

TxA_2

Similar to ADP, TxA_2 is secreted from activated platelets and reinforces cellular activation. TxA_2 is synthesized from arachidonic acid via the cyclooxygenase-1 pathway which is sensitive to treatment with ASA. Significantly, the importance of TxA_2 synthesis inhibition in antiplatelet therapies is illustrated by the efficacy of ASA in the prevention of (recurrent) occlusive thrombotic events in humans, albeit accompanied by an increased risk of bleeding (Michelson 2010).

TxA_2 activates platelets via engagement of the TxA_2 receptor α and β. TxA_2 receptor-deficient mice exhibited

prolonged tail bleeding times and a delayed response to collagen activation in vitro (Thomas et al. 1998). Similarly, ASA treatment led to mildly increased bleeding times in wild-type mice, but, depending on the model and applied dose, had diverging effects on experimental arterial thrombosis (Gruner et al. 2004; Kuijpers et al. 2007; Dutting et al. 2014). For instance, administration of 10 mg/kg body weight ASA to wild-type mice resulted in decreased thrombus stability and increased embolization in $FeCl_3$-injured mesenteric arterioles (Kuijpers et al. 2007), whereas 1 mg/kg body weight ASA affected occlusion times only very mildly in this model and had virtually no effect on the time to stable vessel occlusion in the mechanically injured abdominal aorta (Dutting et al. 2014). By contrast, ASA treatment was accompanied by defective thrombus formation in growth factor receptor-bound protein 2 (Grb2)-deficient mice, whose platelets displayed markedly impaired reactivity to (hem)ITAM-specific receptor stimulation (Dutting et al. 2014). Collectively, these experimental data further emphasize that an important functional redundancy exists between (hem)ITAM and TxA_2-induced GPCR signaling in in vivo thrombus formation.

Monoamines

Platelets take up serotonin (5-hydroxytryptamine, 5-HT) circulating in the periphery and store it in their dense granules. Following platelet activation, serotonin is released and as part of a positive-feedback loop can potentiate cellular activation by acting on G_q-coupled 5-HT_{2A} receptors on the platelet surface (Offermanns 2006). Mice deficient for tryptophan-hydroxylase 1 (Tph1), the rate-limiting enzyme in serotonin biosynthesis in the periphery (Walther et al. 2003a), which consequently also lack peripheral 5-HT, were protected from $FeCl_3$-triggered occlusive thrombus formation in arterioles and displayed significantly prolonged bleeding times (Walther et al. 2003b). Likewise, deficiency of the serotonin transporter 5-HTT in mice (Bengel et al. 1998) which abolishes serotonin uptake into platelets was associated with increased bleeding times and instable thrombus formation in the mechanically injured abdominal aorta or the chemically injured carotid artery (Wolf et al. 2016). Conversely, systemically elevated 5-HT plasma levels through in vivo administration of 5-HT into mice resulted in shortened tail bleeding times and accelerated occlusion rates in $FeCl_3$-injured carotid arteries (Ziu et al. 2012). Studies on serotonin antagonists have long been hampered by the lack of specificity of these agents for the 5-HT_{2A} receptor. Both the prophylactic (prior to injury) and the therapeutic administration of APD791, a selective inverse agonist of the 5-HT_{2A} receptor which does not display functional activity on 5-HT_{2B} and 5-HT_{2C}, have been reported to be protective in a canine model of recurrent coronary thrombosis without affecting hemostatic functions (Przyklenk et al. 2010). However, these studies did not directly address the question whether the protection is solely platelet-dependent or if it can be ascribed in addition to attenuation of serotonin-induced vasoconstriction (Przyklenk et al. 2010). The suitability of 5-HT_{2A} receptor inhibitors to complement existing antithrombotic agents remains to be determined.

Similar to serotonin, epinephrine is a weak platelet agonist which can potentiate existing cellular activation via the α_{2A}-adrenergic receptor that preferentially couples to the G_i-protein G_z on platelets (Nieswandt et al. 2001a; Offermanns 2006). In vitro studies revealed unaltered responses of α_{2A}-deficient platelets to several major platelet agonists, including thrombin, collagen, ADP, and the TxA_2 analog U-46619 (Pozgajova et al. 2006). Interestingly, α_{2A}-deficient mice exhibited reduced thrombus stability and frequent embolization in $FeCl_3$-injured mesenteric arterioles and in the mechanically injured abdominal aorta, which was attributed to reduced potentiation of platelet activation through the α_{2A}-adrenergic receptor at sites of vascular injury (Pozgajova et al. 2006).

The Contact Phase of Blood Coagulation

Recent experimental evidence suggested that the contact phase of blood coagulation, specifically the coagulation factors FXI and FXII, promote arterial thrombosis (Rosen et al. 2002; Wang et al. 2005; Renne et al. 2005; Muller et al. 2011). However, the mechanisms by which platelets activate the intrinsic coagulation pathway remain elusive, and it is currently unclear whether direct interactions between both coagulation factors and platelets indeed occur (e.g., via GPIbα or polyphosphates) (Muller et al. 2009; Heemskerk et al. 2013). Pharmacological inhibition of activated FXII mediated a profound protection from occlusive arterial thrombosis in mice, importantly, without increasing the bleeding risk, which mirrors findings in $F12^{-/-}$ mice and FXII-deficient humans, thus emphasizing the potential of FXII targeting as a novel and safe antithrombotic strategy (Renne et al. 2005; Hagedorn et al. 2010). Of note, several inhibitors of FXI and FXII have been developed, and their in vivo antithrombotic efficacy and safety are currently under preclinical investigation (Kenne and Renne 2014).

Concluding Remarks

Experimental animal models and particularly the use of genetically engineered mice have substantially advanced our understanding of the complex mechanisms underlying thrombus formation in vivo. Genome editing methods

together with significant improvements in intravital microscopy techniques have been instrumental for the refinement of long-standing theories and for the development of novel strategies for the treatment of thrombotic and inflammatory disease conditions. Work in this research area is likely to be significantly accelerated in the future by the large number of mutant mouse strains available from public sources and additionally by the recently introduced CRISPR-Cas9 technique that allows highly efficient genome editing. Constant improvements in resolution enable us now to draw conclusions on the contribution of cell-cell and cell-matrix interactions for the initiation and progression of thrombus growth. Furthermore, these technical advances are increasingly used for the investigation of functions of platelets beyond their well-established role in thrombosis, e.g., for the progression of ischemic stroke or during critical steps of blood/lymphatic vessel separation.

Experimental animal models will remain instrumental for the characterization of novel antiplatelet agents in preclinical research. A significant challenge in this field remains the uncoupling of efficient protection from thrombotic events from undesired side effects on hemostatic functions. This requirement is central to the development of better strategies or the improvement of existing ones for the control of acute and chronic cardio- and cerebrovascular syndromes.

Take-Home Messages

- Intravital microscopy studies in mice have substantially advanced our understanding of the interactions between platelets, other blood cell types, and the vessel wall that controls thrombus growth.
- The tail bleeding time assay is the most frequently used test to assess overall hemostatic functions in mice.
- Widely used mouse in vivo arterial thrombus formation models differ in the size of the vascular bed and the method to induce endothelial denudation by laser, chemical, or mechanical injury.
- Mouse models of thrombosis are indispensable for the development and characterization of antiplatelet drugs.
- State-of-the-art intravital microscopy and tools allowing manipulations of the mouse genome have allowed the identification of novel targets for effective, yet safe, antithrombotic therapy.

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Next-Generation Sequencing in the Study of Platelets

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Abstract

Platelet function disorders (PFDs) arise as a result of defects in platelet formation or function, which can lead to a bleeding diathesis of varying severity, and are often accompanied by additional syndromic features. Well-characterised disorders such as Glanzmann thrombasthenia and Bernard–Soullier syndrome can be diagnosed by extensive platelet phenotyping and confirmed by downstream genetic analysis directed to one or several target genes. However, because of the complexity and heterogeneity of most PFDs, diagnosis and identification of the underlying genetic defect is difficult to obtain. Recent advances in the use of next-generation sequencing (NGS) technologies have facilitated the identification of many novel genetic defects underlying disease. Use of this technology produces a vast amount of genetic information and requires a pipeline of analysis for candidate variant prioritisation. Many studies exclude synonymous and common variants because the most likely candidates are thought to be rare or novel and alter the encoded protein. Identification of the causative variant is greatly aided by the inclusion of multiple affected or unaffected family members. The use of NGS will continue to identify novel genes in association with PFDs and unravel the intracellular mechanisms involved in platelet formation and function, possibly contributing to the development of novel therapies for bleeding disorders and cardiovascular disease.

Platelets play a pivotal role in stemming bleeding following vascular injury. Crucial to thrombus formation is the presence of abundant platelets within the blood stream and their correct functionality. DNA alterations in the genes encoding the proteins that function in a number of cellular processes within a platelet can result in impaired platelet production or function, ultimately leading to a platelet function disorder (PFD). PFDs are characterised by symptoms of excessive bleeding, including easy bruising, epistaxis, gum bleeding and prolonged bleeding following trauma or surgery. It is important to determine whether the disorder is the result of an acquired or inherited defect or is secondary to another disorder. This is usually identified through a detailed clinical history. However, diagnosis of a PFD is difficult if based solely on the clinical symptoms. Following referral, a range of laboratory investigations are carried out to test for coagulopathies such as haemophilia and von Willebrand disease. Platelet number and size are then obtained and further investigations carried out in the form of platelet function tests. These tests include light transmission aggregometry (LTA), which measures platelet aggregation in response to a panel of agonists at a range of concentrations and is usually performed alongside a control subject. In addition, several other techniques can be utilised to provide a full platelet phenotype and include lumiaggregometry to detect dense granule secretion, flow cytometry to detect α-granule secretion and a range of receptor protein levels, and electron microscopy to visualise granule number and morphology (Watson et al. 2013).

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Platelet phenotyping can be used to directly diagnose patients with well-characterised disorders caused by qualitative and/or quantitative impairment of platelet receptors, such as Bernard–Soulier syndrome (BSS) and Glanzmann thrombasthenia, which are confirmed through genetic analysis of target genes. In addition, the results of LTA can suggest less-characterised PFDs associated with defects in platelet receptors, including the $P2Y_{12}$ ADP receptor (Dawood et al. 2012), GPVI (Dumont et al. 2009) and the thromboxane receptor (Hirata et al. 1994; Nisar et al. 2011), directing downstream analysis for identification of the underlying genetic defect.

However, the majority of platelet phenotypes from cases presenting with PFDs do not direct downstream genetic analysis and, therefore, diagnosis of the underlying genetic cause becomes problematic. Potential reasons for this could be gaps in the understanding of the genes involved in specific molecular mechanisms important for platelet function and the redundancy that exists within platelet signalling, leading to difficulties in linking phenotype to genotype.

Use of Next-Generation Sequencing in Identification of Novel Genes Associated with PFDs

The development of next-generation sequencing (NGS) technologies has permitted large-scale simultaneous genetic analysis of a targeted panel of genes, the whole exome or the whole genome. Sequencing of a targeted panel of genes, known or predicted to be involved in a particular disorder, can be more cost-effective than whole exome or genome sequencing. It also provides a higher coverage of the genes of interest and reduces the complexity of analysis. However, as the number of target genes grows for a particular disorder or group of disorders, it becomes more cost-effective to perform whole exome sequencing (WES). Because the majority of disease-causing mutations occur within the coding regions of the genome, WES offers an attractive approach for large-scale genetic analysis. Similarly, because exons account for only 1 % of the genome, the complexity of analysis is reduced compared with that of whole genome sequencing (WGS). However, a complete genetic profile is only provided with WGS, which may identify more novel genetic alterations associated with disease than other types of NGS methods. Interpretation and analysis of WGS data becomes more complex as the amount of information generated increases. All NGS methodologies have proven effective in identifying the underlying genetic causes of many PFDs in cases where the defect was previously unknown. This chapter reviews the novel genes identified as associated with PFDs and discovered using NGS-based methods.

NBEAL2

The earliest benefit of NGS in the diagnosis of bleeding disorders was achieved in 2011 by sequencing the exome of four unrelated patients with gray platelet syndrome (GPS), a recessive disorder characterised by thrombocytopenia, moderate bleeding and a lack of α-granules (Albers et al. 2011). Because the disorder has an autosomal recessive mode of inheritance, homozygous variants or genes in which two or more variants were detected (suggestive of a compound heterozygous mutation) were prioritised and all previously reported single nucleotide variants (SNVs) were removed from analysis. Homozygous or compound heterozygous mutations were identified in the gene *NBEAL2* in all four subjects. Simultaneously, using different approaches, two other groups identified *NBEAL2* as the candidate gene responsible for GPS . Kahr et al. (2011) performed RNA sequencing analysis for the platelets of patients from three families and identified abnormal *NBEAL2* transcripts. Analysis of the *NBEAL2* gene subsequently identified genetic alterations. In a third parallel study, Gunay-Aygun et al. (2011) used genome-wide linkage analysis and homozygosity mapping of 25 individuals from 14 families to identify the disease locus to a 9.4 Mb region. Sequencing of the genes within this region identified mutations in the gene *NBEAL2* in all families, providing further support for the association between this gene and GPS.

The *NBEAL2* gene encodes a Beige and Chediak-Higashi (BEACH)/ARM/WD40 domain protein. The exact role in α-granule biogenesis, however, remains unknown. Knockout of *NBEAL2* in zebrafish leads to impaired thrombocyte formation with spontaneous bleeding (Albers et al. 2011). Reduced α-granule secretion was observed in a knockout mouse model of *Nbeal2*, suggesting a conserved role for the BEACH domain protein in thrombus formation and granule biogenesis. Interestingly, study of $Nbeal2^{-/-}$ mice showed that the α-granules were generated, but not retained, within megakaryocytes and therefore could be a result of alterations in granule biogenesis that trigger granule degradation or improper function (Guerrero et al. 2014). *NBEAL2* expression has also been shown to be upregulated during megakaryocyte maturation, which might also coincide with the production of platelet granules, supporting a role for NBEAL2 in granule biogenesis. Interestingly, $Nbeal2^{-/-}$ mice also display splenomegaly and myelofibrosis, consistent with a GPS phenotype (Guerrero et al. 2014).

The three studies described above identified mutations predicting amino acid alterations that are distributed across the entire protein structure of NBEAL2, resulting in alterations affecting almost all known structural domains (Fig. 1). However, more than half of those identified alterations affect residues at the N-terminus and appear to cluster within the concanavalin A-like lectin (ConA-like) domain. This domain is shared between members of other

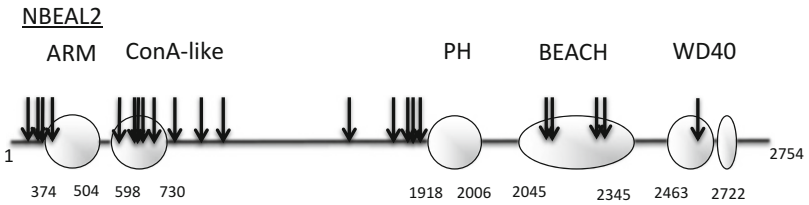

Fig. 1 NBEAL2 structural organisation and variant locations. Representation of the neurobeachin-like 2 (NBEAL2) protein structure of 2754 amino acid residues displaying the Armadillo-type fold (ARM), concanavalin A-like (ConA-like), Pleckstrin homology (PH) and WD40 domains. The locations of variants identified by Albers et al. (2011), Kahr et al. (2011) and Gunay-Aygun et al. (2011) are shown by the *black arrows*. Amino acid sequence and domain architecture are based on accession ID: NP_055990

BEACH domain proteins, including LYST, the causative protein of Chediak–Higashi syndrome (CHS) (Barbosa et al. 1996), and neurobeachin (NBEA), which is associated with myeloma (O'Neal et al. 2009). Lectins are a family of proteins that fuse to cell membranes through the binding and processing of carbohydrates (Chandra et al. 2001). Therefore, the ConA-like domain in BEACH proteins might be involved in oligosaccharide-binding to mediate vesicle fusion events (Burgess et al. 2009). In addition, several mutations were identified in the BEACH domain and a further eight (resulting in a premature stop codon or frameshift mutation) upstream of the BEACH domain. This domain is highly conserved and present in proteins that have a role in vesicle trafficking; however, the exact role remains to be clarified. Structural modelling of the BEACH domain from human NBEA suggests that the BEACH domain interacts with the Pleckstrin homology (PH) domain, which lies 40 amino acids upstream (Jogl et al. 2002). A large groove was also identified in the structure between the PH and BEACH domains, which might facilitate ligand binding (Jogl et al. 2002). In addition, tryptophan–aspartic acid (WD)40 repeat domains, located downstream of the BEACH domain, mediate protein–protein interactions (Neer et al. 1994) and are disrupted as a result of frameshift and stop-gain mutations. The structural sequence of NBEAL2 suggests a role in fusion and protein interactions and, therefore, supports the hypothesis that this BEACH domain protein plays an important role in granule biogenesis and that disturbance of one of several domains impairs protein activity and results in compromised granule biogenesis, as observed in GPS.

RBM8A

Thrombocytopenia with absent radii (TAR) syndrome is characterised by a low platelet count with malformations in limb development (Hall et al. 1969). A 200 kb deletion on chromosome 1q21.1 has been identified in the majority of cases using microarray-based comparative genomic hybridisation (array CGH) (Klopocki et al. 2007). However, the recessive mode of inheritance points to the presence of an additional defect. WES was performed on five patients with TAR syndrome and identified a variant in the 5′ untranslated region (UTR) of the *RBM8A* gene. Sequencing a further 48 TAR syndrome patients who harboured the 1q21.1 deletion revealed the presence of one of two variants located in the *RBM8A* 5′-UTR in 46 patients, with minor allele frequencies (MAFs) of 3.05 % and 0.42 %. It was postulated that the remaining two patients possess an additional defect that remains unidentified. In addition, in two patients with TAR syndrome, but without the 1q21.1 deletion, mutations were identified in the coding region of *RBM8A*. It is therefore postulated that the presence of a single null *RBM8A* allele is not sufficient to cause disease, but that both alleles should be non-functional. This is usually the result of compound inheritance of a rare null allele and one of two rare non-coding SNVs in *RBM8A* that affect regulation (Albers et al. 2012).

RBM8A encodes an RNA-binding protein that forms a component of the splicing-dependent multiprotein exon junction complex (EJC), thought to have a role in pre- and post-mRNA splicing (Kim and Dreyfuss 2001). In the absence of functional RBM8A, key processes such as nuclear mRNA export, translation and nonsense-mediated decay (NMD) are impaired; however, the exact genes mediating these processes via RBM8A remain unknown as do the molecular mechanisms underlying the disease.

ANKRD18A

Inherited thrombocytopenias are a group of highly heterogeneous disorders associated with defects in over 20 genes (Balduini and Savoia 2012; Rao and Songdej 2015). The disorders are characterised by a reduction in platelet number and, often, the presence of abnormally large platelets, with symptoms ranging from mild to severe bleeding tendencies. However, in 50 % of cases, the causative mutation has not been identified. In two siblings of a consanguineous family presenting with severe bleeding symptoms and a low platelet count, WES identified a mutation in the gene *ANKRD18A* in

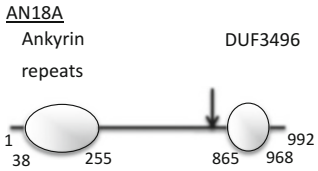

Fig. 2 AN18A structural organisation and variant location. Representation of the Ankyrin repeat domain 18A (AN18A) protein structure of 992 amino acid residues displaying Ankyrin repeats at the N-terminus of the protein and a domain of unknown function (DUF3496) at the C-terminus. The location of the variant identified by Lowe et al. (2012) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_671728.2

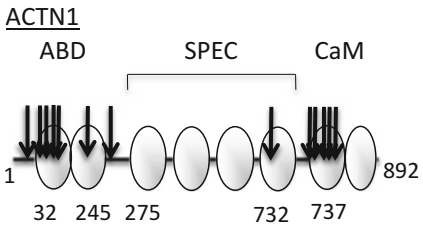

Fig. 3 ACTN1 structural organisation and variant locations. Representation of the actinin, alpha 1 (ACTN1) protein structure of 892 amino acid residues displaying the actin-binding domain (ABD), spectrin repeats (SPEC) and the C-terminal calmodulin domain (CaM). The location of the variants identified by Kunishima et al. (2013), Guéguen et al. (2013) and Bottega et al. (2015) are shown by the *black arrows*. Amino acid sequence and domain architecture are based on accession ID: NP_001093

both affected individuals. In addition to thrombocytopenia, the patients' platelets were also found to have impaired α-granule secretion and integrin activation, suggesting that the bleeding phenotype is the result of a combination of low platelet count and impaired platelet function (Lowe et al. 2012; Watson et al. 2013). The function of the *ANKRD18A*-encoded protein remains unknown but shares conserved regions of Ankyrin repeats and a domain of unknown function (DUF3496) (Fig. 2) with ANKRD26. Genetic mutations are associated with thrombocytopenia, α-granule deficiency and a predisposition to leukaemia (Al Daama et al. 2013; Noris et al. 2015). The mutations described in *ANKRD26* are located in the 5′-UTR regulatory region (Noris et al. 2015) or in the Ankyrin repeats, which is likely to affect protein binding. The mutation identified in *ANKRD18A* is an in-frame deletion upstream of DUF3496 (Al Daama et al. 2013); however, the effect of this mutation on protein function has yet to be investigated.

ACTN1

In six Japanese families presenting with mild or absent bleeding diatheses, reduced platelet counts, increased platelet size and anisocytosis, WES was applied in a total of 11 affected and 10 unaffected individuals (Kunishima et al. 2013). Following the removal of previously reported variants and prioritisation based on in silico predictions (assuming a dominant mode of inheritance), six genes were predicted to be pathogenic in more than one family. Calculation of significance based on the number of variations identified and the size of the coding region showed that *ACTN1* was the most significant and associated with disease in three families. Subsequently, the *ACTN1* gene was analysed using Sanger sequencing in a further seven families, three of which were found to harbour *ACTN1* mutations that co-segregate with disease (Kunishima et al. 2013). Accordingly, in a large French pedigree consisting of 55 members, 26 with inherited macrothrombocytopenia, a combination of linkage analysis and targeted NGS identified a single causative variant to lie within the gene *ACTN1* (Guéguen et al. 2013). A further study identified ten *ACTN1* mutations in thrombocytopenia patients, following WES analysis in seven index cases and another 128 cases using conventional sequencing methods. Of the ten mutations, seven resulted in novel amino acid substitutions (Bottega et al. 2015).

The *ACTN1* gene encodes the protein α-actinin, an actinin cross-linking protein involved in cytoskeletal organisation. In vitro expression studies demonstrated that most mutations caused cytoskeletal disorganisation, with a reduction in the number of proplatelets formed and an increase in platelet swelling, accounting for the enlarged platelets observed (Guéguen et al. 2013; Kunishima et al. 2013; Bottega et al. 2015). α-Actinin exists as a dimer with two functional domains, an actinin-binding domain (ABD), composed of two calponin homology regions, and a C-terminal calmodulin-like domain (CaM) made up of two EF-hand Ca^{2+}-binding motifs. Most of the identified *ACTN1* mutations across the three studies predict amino acid substitutions that reside within the ABD or the CaM domains (Fig. 3). Interestingly, a variant located outside of these functional domains, in a spectrin repeat, did not cause any alteration to cytoskeletal structure (Bottega et al. 2015), which supports the importance of actinin binding and Ca^{2+} binding in the function of α-actinin in mediating platelet formation.

FLI1 and *RUNX1*

The UK Genotyping and Phenotyping of Platelets (UK-GAPP) multicentre study aimed to recruit patients with PFDs and identify the underlying genetic defects responsible for the variety of platelet phenotypes (Watson et al. 2013). To date, over 500 patients have been recruited and platelet defects identified in 60 % of cases. LTA was

used to assess platelet function in patients with a suspected PFD. The differential responses in aggregation, using a range of agonists, allowed patients to be divided into diagnostic sub-groups (Dawood et al. 2012). The majority of patients diagnosed with PFDs fell into one of three sub-groups: dense granule secretion defect, Gi-signalling defect and thromboxane defect. Performing genetic analysis on patients grouped by platelet phenotype should increase the chances of identifying the causative variants in such a complex group of disorders. NGS of a targeted gene panel consisting of over 260 genes, known or predicted to have a role in platelet formation or function (Jones et al. 2012; Stockley et al. 2013), was undertaken in 13 index cases with severe defects in dense granule secretion. After the removal of duplicates and focussing on novel variants only, mutations were identified in *FLI1* or *RUNX1* that co-segregate with disease in 6 out of the 13 families (Stockley et al. 2013). Additional studies have since utilised the power of NGS technologies to identify *FLI1* aberrations. Sequencing of a targeted panel of 19 genes identified a homozygous variant in the *FLI1* gene in a patient with moderate thrombocytopenia from a family of consanguineous marriage (Stevenson et al. 2015). Further utilisation of WES also identified another *FLI1* variant in a patient with thrombocytopenia (Poggi et al. 2015). Interestingly, however, neither of these patients was found to have any defect in granule secretion (Stevenson et al. 2015; Poggi et al. 2015).

FLI1 and RUNX1 are transcription factors that cooperate during the late stages of megakaryopoiesis (Huang et al. 2009; Tijssen and Ghevaert 2013). Hemizygous loss of FLI1 has been associated with Paris–Trousseau syndrome (PTS), in which patients bleed excessively as a result of thrombocytopenia and the presence of abnormally large α-granules (Breton-Gorius et al. 1995; Favier et al. 2003). In accordance, the presence of abnormally large α-granules was observed in a subset of platelets from patients with *FLI1* genetic alterations (Stevenson et al. 2015; Leo et al. 2015a, b). However, it remains unknown how FLI1 is involved in the process of granule formation and function and how the mutations identified lead to a defect in dense granule secretion. The authors showed that the missense changes abolish transcriptional regulation of the FLI1 protein (Stockley et al. 2013; Stevenson et al. 2015) and, therefore, downregulation of target genes (yet to be identified) could be responsible for this phenotype. Furthermore, some patients with *FLI1* defects appear to have syndromic features such as eczema and alopecia with severe bleeding (Stockley et al. 2013), whereas others possess a mild bleeding tendency with no additional symptoms (Stevenson et al. 2015; Poggi et al. 2015). Therefore, an understanding of the genes regulated by FLI1 and the extent to which their regulation is compromised in each case is required to unravel the mechanism by which FLI1 functions.

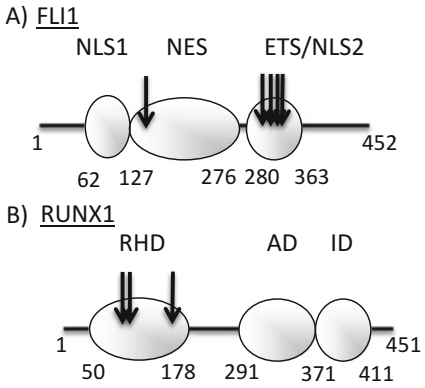

Fig. 4 FLI1 and RUNX1 structural organisation and variant locations. (**a**) Representation of the Friend leukaemia integration 1 transcription factor (FLI1) protein structure of 452 amino acid residues displaying nuclear localisation signals NLS1 and NLS2, nuclear export signal (NES) and the ETS DNA-binding domain. The location of the variants identified by Stockley et al. (2013), Stevenson et al. (2015), and Poggi et al. (2015) are shown by the *black arrows*. Amino acid sequence and domain architecture is based on accession ID: NP002008 and evidence from Hu et al. (2005). (**b**) Representation of the Runt-related transcription factor 1 (RUNX1) protein structure of 451 amino acid residues displaying the RUNT homology domain (RHD), activation domain (AD) and the inhibitory domain (ID). The location of the variants identified by Stockley et al. (2013) are shown by the *black arrows*. Amino acid sequence and domain architecture are based on accession ID: NP_001745

The FLI1 protein comprises two nuclear localisation signals (NLS1 and NLS2), a nuclear export signal (NES) and the ETS DNA-binding domain (Hu et al. 2005). It is clear that the ETS domain is of great importance to the function of FLI1, with four of the five variants located within this domain (Fig. 4a) abolishing transcriptional activity. These variants include those identified in three patients with severe bleeding symptoms and defects in dense granule secretion (Stockley et al. 2013), whereas the variant located within the NES appears to be less pathogenic (Poggi et al. 2015).

Mutations in *RUNX1* have been associated with a predisposition to acute myeloid leukaemia (AML) (Nurden et al. 2012); however, in the patients described in the study by Stockley et al. (2013) no family history of malignancy was evident. It has been demonstrated that *RUNX1* mutations lead to a reduction in the expression of several genes, most notably *MYL9* (Sun et al. 2007) as well as *PRKCQ* (Jalagadugula et al. 2011), *ALOX12* (Kaur et al. 2010) and *PLDN* (Mao et al. 2015). These genes have been shown to play a crucial role in dense-granule secretion, thereby explaining how alterations in the transcription factor could result in the platelet phenotype observed. The variants identified in the study by Stockley et al. (2013) were located to the RUNT domain (Fig. 4b), which is essential for DNA binding and therefore likely to impair transactivational capacity. The majority of mutations have also been

identified in the same domain in patients with a predisposition to AML (Michaud 2002). Therefore, the location of the alteration does not appear to discriminate between a PFD and malignancy, but is more probably caused by the nature of the mutation, with more severe consequences in protein activity as a result of homozygous or dominant-negative mutations increasing the risk of malignancy (Matheny et al. 2007). This is supported by a study that compared the transcriptome of two pedigrees harbouring *RUNX1* mutations, with and without a history of malignancy, which identified an association of absent *NR4A3* transcript levels with malignancy. Reduced, but detectable, levels lead to a PFD and symptoms of excessive bleeding only (Bluteau et al. 2011).

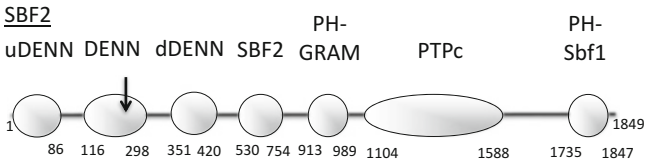

Fig. 5 SBF2 structural organisation and variant location. Representation of the SET binding factor (SBF2) protein structure of 1849 amino acid residues displaying the domain always found upstream of DENN domain (uDENN), the DENN domain, the domain always found downstream of DENN domain (dDENN), SBF2 domain, PH-GRAM_MTMR13 domain, the protein tyrosine phosphatases catalyse domain (PTPc) and a C-terminal PH-Sbf1_hMTMR5. The location of the variant identified by Abuzenadah et al. (2013) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_112224

SBF2

A rare case of thrombocytopenia accompanied by reduced pigmentation was reported by Abuzenadah et al. (2013). Other platelet disorders have been reported to accompany the feature of albinism, as in the case of Griscelli syndrome (Griscelli et al. 1978), Hermansky–Pudlak syndrome (HPS) (Hermansky and Pudlak 1959) and CHS (Barbosa et al. 1996). However, the presence of a normal platelet count is observed in HPS and CHS, and with no immunological or neurological defects in the patient described by Abuzenadah et al. (2013), the disorder is unlikely to be Griscelli syndrome. Therefore, the patient and the unaffected father underwent exome sequence analysis, assuming a recessive mode of inheritance because both parents presented with no symptoms. Focussing on homozygous variants, predictions of pathogenicity using bioinformatic tools and the removal of previously reported variants, a single homozygous mutation in the gene *SBF2* was identified and later verified in an affected sibling. The mutation was heterozygous in both parents and absent in an unaffected sibling (Abuzenadah et al. 2013). SBF2 is a pseudo-phosphatase, part of the myotubularin family of lipid phosphatases, and has been associated with Charcot–Marie–Tooth 4 type B2 (CMT4B2) neuropathy (Bird 2014; Baets et al. 2011).

The mutation identified in association with CMT4B2 deletes the dDENN domain (Senderek 2003), whose exact function remains unknown. In contrast, the mutation associated with a novel form of thrombocytopenia was located in the DENN domain of the SBF2 protein (Fig. 5), which mediates interactions with the RAB family of small GTPases, including Rab27a and Rab28 (Marat et al. 2011). Given the known roles of the interacting partners in vesicle transport, it is possible that the patient's platelets exhibit an additional defect in granule biogenesis; however, this could not be tested because of the low platelet count. Further work is required to understand the role of *SBF2* in platelet production and how mutations in this gene can lead to the varied phenotypic features observed.

THPO

Aplastic anaemia is a condition that arises as a result of defects in all blood cell lineages, leading to symptoms of increased bleeding caused by thrombocytopenia, susceptibility to infection as a result of deficient leukocytes, and anaemia through inefficient red cells (Brodsky and Jones 2005). In a family with aplastic anaemia, array CGH was used initially to identify runs of homozygosity and copy number variations (CNV) to determine a candidate gene locus; however, no loci were identified. WES was subsequently undertaken, focussing on homozygous variants in the coding regions shared between two affected siblings and not previously reported on databases such as dbSNP or 1000 genomes. Seven homozygous variations were identified in seven genes. Confirmation and screening for the presence of these variants in an unaffected sibling and one parent using Sanger sequencing reduced the number of candidate defects to three. Use of in silico predictions strongly predicted a variant in the gene *THPO* to be highly pathogenic, whereas the other two variants were predicted to be benign (Dasouki et al. 2013).

THPO encodes thrombopoietin (TPO), a ligand for the c-Mpl receptor required for haematopoietic lineage differentiation (Hitchcock and Kaushansky 2014). The variant is located in the receptor binding domain (RBD) (Fig. 6); structural modelling of the variant suggests disturbance to the region. In vitro studies demonstrated a reduced ability to maintain cell proliferation, which probably contributes to the lower number of maturing blood cells. Knockout studies in mice have previously demonstrated an association of *Thpo* and *Mpl* with a decrease in the formation of all blood cell types (Alexander et al. 1996; Carver-Moore et al. 1996; Bunting et al. 1997; Dorsch et al. 1999).

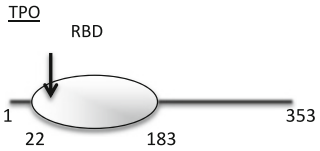

Fig. 6 TPO structural organisation and variant location. Representation of the thrombopoietin (TPO) protein structure of 353 amino acid residues displaying the receptor binding domain (RBD) and the N-terminal half of the protein. The location of the variant identified by Dasouki et al. (2013) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_000451

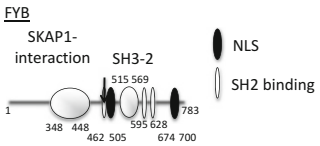

Fig. 7 FYB structural organisation and variant location. Representation of the FYN binding protein (FYB) protein structure of 783 amino acid residues displaying the Interaction with SKAP1 domain, three SH2-binding domains (*thin white ovals*), two nuclear localisation signals (NLS, *black ovals*) and the SH3-2 domain. The location of the variant identified by Hamamy et al. (2014) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_955367

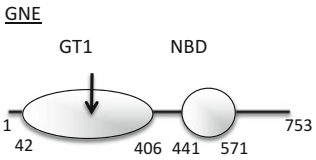

Fig. 8 GNE structural organisation and variant location. Representation of the glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase (GNE) protein structure of 753 amino acid residues displaying the GT1_UDP-GlcNAc_2-epimerase domain (GT1) and the nucleotide binding domain_sugar-kinase_HSP70_actin (NBD). The location of the variant identified by Izumi et al. (2014) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_001121699

FYB

WES was carried out in three affected and nine unaffected members of a consanguineous family with thrombocytopenia and small platelets. The analysis pipeline focussed on homozygosity resulting from the autosomal recessive mode of inheritance, taking into account that parental DNAs both contain one copy of the variant that is present in the homozygous state in affected individuals. Further prioritisation was achieved by removal of synonymous variants and those with a MAF of over 0.02. A single variant, a 2-bp deletion leading to a premature stop codon, was identified as segregating with disease in the gene *FYB* (Hamamy et al. 2014). Several groups have demonstrated a defect in platelet function resulting from *Fyb* knockout in mice, including an increased bleeding tendency (Kasirer-Friede et al. 2007) with mild thrombocytopenia, a reduction in splenic T cells and thymocyte number (Peterson et al. 2001) and enlarged spleens, which may reflect the removal of platelets from circulation (Jarvis et al. 2011).

FYB encodes the FYN binding protein, which associates with FYN via the SH2-binding domains and plays a role in intracellular signalling (da Silva et al. 1993). The frameshift deletion identified in the family reported by Hamamy et al. (2014) lies within one of several SH2-binding domains, altering the downstream sequence including two more SH2-binding domains (Fig. 7). Impaired affinity for FYN is likely to result in impaired intracellular signalling, which may alter megakaryocyte maturation or platelet formation and account for a low platelet count and small platelet size. In addition, FYN binding protein was shown to play a role in signalling mediated by the $\alpha_{IIb}\beta_3$ receptor, promoting platelet spreading and thrombus stabilisation under shear stress conditions (Kasirer-Friede et al. 2007). Therefore, in addition to a reduced platelet count, impaired platelet activation could also be responsible for the bleeding phenotype observed in $Fyb^{-/-}$ mice and patients with *FYB* mutations.

GNE

In a family presenting with thrombocytopenia accompanied by myopathy with rimmed vacuoles, WES analysis of two affected family members, in combination with array CGH, identified mutations in two candidate genes, *GNE* and *FLNB*, following a pipeline to exclude common variants and retain homozygous or compound heterozygous variants in accordance with the recessive mode of inheritance (Izumi et al. 2014). A compound heterozygous mutation was identified in the gene *GNE* in both affected family members. Each variant has been reported previously in association with distal myopathy with rimmed vacuoles (DMRV) (Tomimitsu et al. 2002, 2004).

GNE encodes the protein glucosamine (UDP-*N*-acetyl)-2-epimerase/*N*-acetylmannosamine kinase, an enzyme that produces sialic acid, which modifies surface proteins to influence a range of cellular functions that include cell signalling, adhesion and migration (Kelm et al. 1994; Bassagañas et al. 2014). The mutation is located in the GT1_UDP-GlcNAc_2-epimerase (GT1) domain of the encoded protein (Fig. 8), a critical region for the hydrolysis of UDP-GlcNAc to produce UDP-*N*-acetylmannosamine (UDP-ManNAc), a precursor to sialic acids (Marchler-Bauer et al. 2014). Therefore, the compound heterozygous mutation in *GNE* is a strong candidate as cause of the myopathic phenotype observed in these patients.

Additionally, platelets deficient in sialic acid have been shown to increase platelet clearance, resulting in thrombocytopenia (Sørensen et al. 2015). However, because thrombocytopenia has not been reported in previous cases of myopathy with rimmed vacuoles caused by genetic alterations in *GNE* (Tomimitsu et al. 2002, 2004; Nishino et al. 2015), the effect of the mutation in *FLNB* identified in the affected family members has yet to be determined. It may play a similar role to its homologue *FLNA*, which is associated with thrombocytopenia (Fox et al. 1998; Nurden et al. 2011), and so remains a plausible candidate.

PRKCAG

In a consanguineous family with macrothrombocytopenia, abnormal surface expression of GPIb-XI-V complex and αIIβ3 and defective α-granule secretion, whole exome data for two affected and three unaffected family members were scrutinised to identify the causative defect. Because the proband was of consanguineous marriage and neither parent presented with any bleeding symptoms, an autosomal recessive mode of inheritance was assumed and heterozygous variants removed. Further prioritisation was achieved following the removal of synonymous variants and focussing on those with a MAF of less than 0.01 (Manchev et al. 2014). Two variants were identified that segregated with disease, altering the genes *GNE* and *PRKCAG*.

PRKCAG encodes the gamma isoform of the catalytic subunit of cAMP-dependent PKA, which in turn phosphorylates filamin A (FLNA) (Jay et al. 2004) and GPIBβ (Bodnar et al. 2002), both being associated with macrothrombocytopenia. *GNE* mutations have been associated with myopathy, as described above (Eisenberg et al. 2001), but the patients exhibit no such phenotypic features. Therefore, the presence of a mutation in *PRKCAG* appeared to be the more likely candidate as a cause of the observed phenotype. Indeed, it was shown that FLNA levels were markedly reduced in the patients' platelets compared with control platelets, suggesting a lack of FLNA phosphorylation by PKA, which would otherwise protect the protein from degradation (Chen and Stracher 1989). FLNA is responsible for connecting platelet receptors to the cytoskeleton, which could explain the absence of platelet receptor activation (Nurden et al. 2011). Furthermore, megakaryocyte maturation was not affected by the mutation; however, the mutation did lead to a marked reduction in proplatelet formation compared with the wild type. The mutation identified lies within the serine/threonine/dual specificity protein kinase catalytic (STKc) domain (Fig. 9) and adjacent to an ATP-binding site. It could therefore conceivably alter the kinase activity of the protein towards phosphorylate targets such as FLNA.

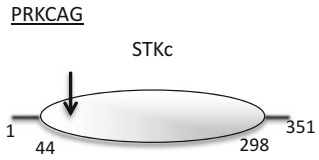

Fig. 9 PRKCAG structural organisation and variant location. Representation of the catalytic subunit C gamma of the cAMP-dependent protein kinase (PRKCAG) protein structure of 351 amino acid residues displaying a single Serine/threonine/dual specificity protein kinase, catalytic domain (STKc). The location of the variant identified by Manchev et al. (2014) is shown by the *black arrow*. Amino acid sequence and domain architecture are based on accession ID: NP_002723

ETV6

Two independent research groups have identified *ETV6* mutations as being associated with platelet production. In the study by Noetzli et al. (2015), WES was undertaken in five affected members of a single pedigree who presented with symptoms of mild to moderate bleeding, with two members developing B-cell precursor acute lymphoblastic leukaemia. A reduction in platelet number was detected, with a fraction containing elongated α-granules and an increased mean red blood cell volume. Analysis of the whole exome data identified a single gene defect that segregated with thrombocytopenia and a high red cell volume following the removal of synonymous variants and those with a MAF of over 1 % in the general population. The *ETV6* gene was subsequently screened in a further 23 families with thrombocytopenia, high mean red cell volume and increased incidence of leukaemia using conventional sequencing methods. *ETV6* genetic alterations were identified in two of the 23 families, with a predisposition to malignancy in one family (Noetzli et al. 2015).

In a parallel study, Zhang et al. (2015) analysed WES data from four affected and one unaffected family members presenting with similar features to the families described by Noetzli et al. (2015). Variants were filtered on the basis of an autosomal dominant mode of inheritance and removal of common and synonymous SNVs, which identified five variants segregating with disease. Sanger sequencing of all five genes in a further two unaffected family members revealed complete segregation of only the *ETV6* variant with disease. Targeted sequencing in 55 individuals then identified a further two families with *ETV6* mutations presenting with thrombocytopenia and haematologic malignancy (Zhang et al. 2015).

ETV6 is a transcription factor that binds to and represses the expression of target genes via the ETS domain (Kwiatkowski et al. 1998; Chakrabarti and Nucifora 1999; Fenrick et al. 1999; Lopez et al. 1999) and requires dimerisation in order to function (Green et al. 2010). Four

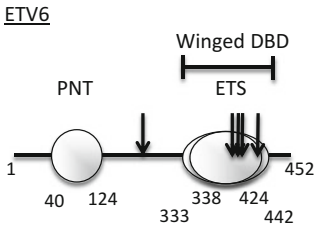

Fig. 10 ETV6 structural organisation and variant locations. Representation of the Ets variant 6 (ETV6) protein structure of 452 amino acid residues displaying a pointed domain (PNT) and an ETS DNA binding domain, within the winged helix-turn-helix DNA binding domain (Winged DBD). The location of the variants identified by Noetzli et al. (2015) and Zhang et al. (2015) are shown by the *black arrows*. Amino acid sequence and domain architecture are based on accession ID: NP_001978.1

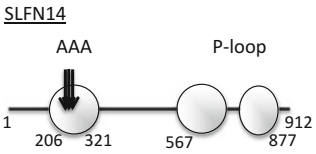

Fig. 11 SLFN14 structural organisation and variant location. Representation of the Schlafen family member 14 (SLFN14) protein structure of 912 amino acid residues displaying an ATPase-AAA domain (AAA) and a P-loop containing a nucleoside triphosphate hydrolase domain (P-loop). The location of the variants identified by Fletcher et al. (2015) are shown by the *black arrows*. Amino acid sequence and domain architecture are based on accession ID: NP_001123292

of the five mutations identified in patients with thrombocytopenia are located in the ETS domain and another within the central region of the protein (Fig. 10). Although the ETS domain is a highly conserved region across ETS transcription factor family members and crucial for DNA binding (Degnan et al. 1993), previous studies have identified mutations throughout the entire locus that are causative of various types of malignant haematologic disorders and solid tumours, suggesting important roles for regions spanning the entire protein. In support of this, the N-terminal pointed (PNT) domain is responsible for homodimerisation (Tognon et al. 2004) and the central regulatory region is required for post-translational modification and is essential for the repressive function of the protein (Zhang et al. 2015; Noetzli et al. 2015); therefore, alterations to any of these functions is likely to impair ETV6 activity. However, the majority of the mutations identified in families with thrombocytopenia accumulate within the ETS domain, with a single mutation in the central region, all of which function in a dominant-negative manner to decrease transcriptional repression, impair nuclear localisation and alter megakaryocyte maturation (Zhang et al. 2015; Noetzli et al. 2015).

The exact role of ETV6 in haematopoiesis and the association with malignancy in humans, mouse and zebrafish (Rasighaemi et al. 2014) requires further clarification, but bears remarkable similarities to that of RUNX1 (Song et al. 1999). Interestingly, ETV6 is known to bind to and repress FLI1 transcriptional activity (Kwiatkowski et al. 1998), which poses the questions of whether FLI1 activity is increased in these patients and what the underlying consequence is of increased transactivation capacity of FLI1.

SLFN14

As part of the UK-GAPP study, WES was carried out for three affected family members with thrombocytopenia, presenting with symptoms of excessive bleeding with a dominant mode of inheritance. Platelet phenotyping revealed that, in addition to a reduced platelet count, dense granule secretion and platelet aggregation in response to several agonists was impaired. Following removal of synonymous and previously identified variants, four remained that segregated with disease in this family. However, confirmation via Sanger sequencing left just two variants that were shared between all affected members. Cross-referencing both genes to the exomes of 35 index cases with thrombocytopenia and/or secretion defects recruited to the GAPP study highlighted *SLFN14* as a novel gene with an important role in the formation and function of platelets. Heterozygous *SLFN14* mutations were identified in three families with thrombocytopenia and defects in dense granule secretion (Fletcher et al. 2015). The patients' platelets were shown to contain fewer dense granules per platelet than those of a control subject, possibly accounting for the reduction in dense granule secretion. A reduction in Schlafen family member 14 (SLFN14) protein levels was also observed as a result of the mutations identified, with a 65–80 % reduction in SLFN14 protein levels following overexpression in transfected HEK293T cells and 66–76 % reduction in the patients' platelets, suggesting a dominant-negative effect.

Schlafen family members have been shown to play a role in cell proliferation and differentiation (Schwarz et al. 1998; Geserick et al. 2004; Brady et al. 2005; van Zuylen et al. 2011; Kuang et al. 2014) and are expressed in various cells of the haematopoietic lineage, including platelets (Rowley et al. 2011; Kim et al. 2014). The exact role of *SLFN14* and how mutations in this gene lead to the observed phenotype, however, remains to be elucidated. Interestingly, the three mutations identified in the three families are predicted to cause consecutive amino acid substitutions that lie within the ATPase-AAA domain (Fig. 11), a conserved region for all members of the Schlafen family. The domain is important for ATP/GTP hydrolysis (Hanson and Whiteheart 2005) and is present in proteins that play a number of cellular functions, including cell-cycle regulation and intracellular transport (Kedzierska 2006). The three consecutive residues

are also highly conserved across multiple species, suggesting high importance with regards to the function of SLFN14 (Fletcher et al. 2015).

Limitations of Next-Generation Sequencing

To facilitate prioritisation of candidate gene defects where NGS was applied, the majority of studies used a pipeline that excluded synonymous variants and variants with a MAF of over 0.01, as disease-causing mutations are mainly rare and alter the encoded protein. Furthermore, for heterogeneous disorders, multiple family members are vital for identification of causative genetic defects, with the addition of each family member reducing the number of candidate variants by 50 % (Gilissen et al. 2012). Identification of causative genes can be more difficult in a cohort of index cases where bleeding is the only clinical feature, but functional annotation analysis can aid the prioritisation of genes on the basis of functions within similar pathways (Leo et al. 2015a, b). Focussing on the whole exome and exclusion of synonymous variants could miss pathogenic mutations that would cause alterations in the splicing process or gene regulation (Gilissen et al. 2012). Other genetic mechanisms may have also been overlooked by NGS approaches, such as copy number variation and large insertions and deletions. If WES does not identify a causative mutation, those particular studies should include synonymous variants within the pipeline of analysis and consider the use of WGS. RNA expression profiles via RNA sequencing can also help narrow the search for candidate genes.

The Future for Use of Next-Generation Sequencing in Diagnosis of Platelet Disorders

The assignment of novel genetic aetiology to PFDs in the 1980s progressed relatively slowly and relied on extensive platelet phenotypic data that directed downstream genetic analysis (Table 1, Fig. 12). The mid-1990s saw a dramatic increase in the rate of discovery as a result of utilisation of linkage analysis, mapping genetic loci that segregate with disease. This technology assumes that, of the numerous SNVs or genetic markers distributed across the genome, those that lie within close proximity to the disease-causing allele are also inherited as a result of genetic linkage and therefore map the chromosomal location of the disease-causing gene(s) (Pulst 1999). This technique has been extremely powerful in gene discovery, particularly for cases where there are strong phenotypic features, such as Wiskott–Aldrich Syndrome (WAS) (Donner et al. 1988) and MYH9-related disorders (Seri et al. 2000; Martignetti et al. 2000; Cusano et al. 2000) (Table 1). At the turn of the century, linkage was still being utilised as the main method for gene discovery and maintained a steady rate of novel gene identification. However, the appearance of NGS technologies in the last 5 years has further enhanced the rate of gene discovery (Fig. 12), especially in more complex and heterogeneous disorders. This was sometimes achieved through a combination of linkage analysis with NGS (Guéguen et al. 2013; Stevenson et al. 2015); however, the sole use of WES in family studies is becoming increasingly more common for the identification of disease-causing variants (Table 1 and Fig. 12). As the cost of WGS continues to fall, it is likely that we will see a rise in its use for variant identification. Although the amount of data generated poses additional challenges for analysis and interpretation, combining this information with detailed laboratory and clinical phenotypes in large patient cohorts can ultimately facilitate identification of the underlying genetic causes in numerous cases. This approach is being utilised by the BRIDGE Bleeding and Platelet Disorder (BPD) study through the use of the Human Phenotype Ontology (HPO) database (Köhler et al. 2014), which relies on a library of terms describing phenotypes. The use of an algorithm phenotypically clusters cases based on HPO terms and aids identification of candidate genes on the basis of those shared within the cluster (Westbury et al. 2015). This approach is supported by the fact that 60 % of cases present with features additional to bleeding, with a median of seven HPO terms per case, and have replicated the findings of *MYH9*, *ACTN1* and*GFI1B* gene defects in patients that form clusters. In addition, a novel gene (*PKC*) has been identified in association with GPS-like syndrome characterised by macrothrombocytopenia, bleeding, myelofibrosis, facial abnormalities and bone pathologies (Wijgaerts 2015). As a branch of the BRIDGE–BPD study, the ThromboGenomics project aims to provide a platform for routine diagnosis of rare BPDs. The platform permits NGS analysis and rapid variant-identification for 77 genes associated with platelet and coagulation disorders, providing an efficient and affordable approach for genetic diagnosis (https://haemgen.haem.cam.ac.uk/thrombogenomics). It is also likely that the ThromboGenomics project will utilise phenotypic characteristic data through the HPO database to aid diagnosis of BPDs. Through NGS technologies, novel genes will continue to be identified in association with disease. Combining WGS data with RNA expression profiles and proteomics may help to stratify candidate gene identification and create a more complete picture of disease cause.

Table 1 The history of gene discovery associated with PFDs

Gene	Method of detection	Disorder	Reference
WAS	Linkage analysis	Wiskott–Aldrich Syndrome	Donner et al. (1988)
ITGA2B, ITGB3	Platelet phenotyping and restriction analysis	Glanzmann thrombasthenia	Bray and Shuman (1990)
GPIBA	Platelet phenotyping and Sanger sequencing	Bernard–Soullier Syndrome (BSS)	Ware et al. (1990)
GP9	Platelet phenotyping and Sanger sequencing	BSS	Wright et al. (1993)
TBXA2R	Platelet phenotype and conventional sequencing	Thromboxane A2 receptor defect	Hirata et al. (1994)
11q23 deletion	Cytogenetic analysis and chromosome painting	Paris–Troussaeu syndrome	Breton-Gorius et al. (1995)
LYST	Sanger sequencing	Chediak–Higashi syndrome (CHS)	Barbosa et al. (1996)
HPS1	Linkage analysis	Hermanksy–Pudlak syndrome (HPS) 1	Oh et al. (1996)
FLNA	Linkage analysis	Periventricular heterotopia with macrothrombocytopenia	Fox et al. (1998)
HPS2/AP3	Sanger sequencing	HPS2	Angelica et al. (1999)
MYO5A, RAB27A, MLPH	Linkage analysis	Griscelli syndrome	Ménasché et al. (2000), Pastural et al. (2000)
HOXA11	Sanger sequencing	Radioulnar synostosis with amegakaryocytic thrombocytopenia	Thompson and Nguyen (2000)
MYH9	Linkage analysis	Thrombocytopenia with varied syndromic features	Seri et al. (2000), Martignetti et al. (2000), Cusano et al. (2000)
GATA-1	Sanger sequencing	GATA-1 related thrombocytopenia	Nichols et al. (2000)
ABCG5, ABCG8	Sanger sequencing	Mediterranean stomatocytosis/ macrothrombocytopenia	Berge et al. (2000)
MPL	Sanger sequencing	Amegakaryocytic thrombocytopenia	Ballmaier et al. (2001)
GPIBB	Restriction analysis and Sanger sequencing	BSS	Kunishima et al. (2001)
HPS3	Linkage analysis	HPS3	Anikster et al. (2001)
HPS4	Sanger sequencing	HPS4	Suzuki et al. (2002)
HPS5, HPS6	Sanger sequencing	HPS5/HPS6	Zhang et al. (2003)
HPS7/DTNBP1	Sanger sequencing	HPS7	Li et al. (2003)
UNC13D/ MUNC13-14	Linkage analysis and high-resolution SNP genotyping	Familial hemophagocytic lymphohistiocytosis (FHL) 3	Feldmann et al. (2003)
VPS33B	Linkage analysis	Arthrogryposis–renal dysfunction–cholestasis	Gissen et al. (2004)
STX11	Linkage analysis	FHL4	zur Stadt et al. (2005)
HPS8/ BLOC1S3	Linkage analysis	HPS8	Morgan et al. (2006)
PRF1	Linkage analysis	FHL2	Jabado et al. (1999), Trizzino et al. (2008)
TBXAS1	Linkage analysis	Ghosal syndrome	Geneviève et al. (2008)
PLA2G4A	Sanger sequencing	Cytosolic phospholipase A2 deficiency	Adler et al. (2008)
CYCS	Linkage analysis	Thrombocytopenia	Morison et al. (2008)
FERMT3	Linkage analysis	Leukocyte adhesion deficiency type III syndrome	Kuijpers et al. (2008)
GP6	Platelet phenotyping and Sanger sequencing	GPVI collagen receptor defect	Dumont et al. (2009)
STXBP2	Linkage analysis	FHL5	zur Stadt et al. (2009)
TUBB1	Sanger sequencing	Macrothrombocytopenia	Kunishima et al. (2009)

(continued)

Table 1 (continued)

Gene	Method of detection	Disorder	Reference
PLAU	Linkage analysis	Quebec syndrome	Paterson et al. (2015)
HPS9/PLDN	Sanger sequencing	HPS9	Cullinane et al. (2011)
ANKRD26	Linkage analysis	ANKRD26-related thrombocytopenia	Pippucci et al. (2011)
NBEAL2	WES	Grey platelet syndrome (GPS)	Albers et al. (2011), Gunay-Aygun et al. (2011), Kahr et al. (2011)
P2RY12	Platelet phenotyping and Sanger sequencing	$P2Y_{12}$ ADP receptor defect	Dawood et al. (2012)
RBM8A	WES	Thrombocytopenia with absent radii	Albers et al. (2012)
ANKRD18A	WES	Thrombocytopenia	Lowe et al. (2012)
ACTN1	WES	Macrothrombocytopenia	Kunishima et al. (2013)
GFI1B	Linkage analysis + NGS	Macrothrombocytopenia with reduced α-granules	Stevenson et al. (2013)
FLI1, RUNX1	NGS	Dense granule secretion defect	Stockley et al. (2013)
SBF2	WES	Thrombocytopenia with reduced pigmentation	Abuzenadah et al. (2013)
THPO	WES	Aplastic anaemia	Dasouki et al. (2013)
FYB	WES	Microthrombocytopenia	Hamamy et al. (2014)
GNE	WES	GNE myopathy with thrombocytopenia	Izumi et al. (2014)
PRKACG	WES	Macrothrombocytopenia	Manchev et al. (2014)
ETV6	WES	Thrombocytopenia with malignancy	Zhang et al. (2015), Noetzli et al. (2015)
SLFN14	WES	Thrombocytopenia with dense granule secretion defects	Fletcher et al. (2015)
MKL1, PF4	WES	Thrombocytopenia	Johnson et al. (2016)
PKC	WGS and HPO	GPS-like syndrome	Wijgaerts (2015)

NGS next generation sequencing, *WES* whole exome sequencing, *WGS* whole genome sequencing, *HPO* human phenotype ontology

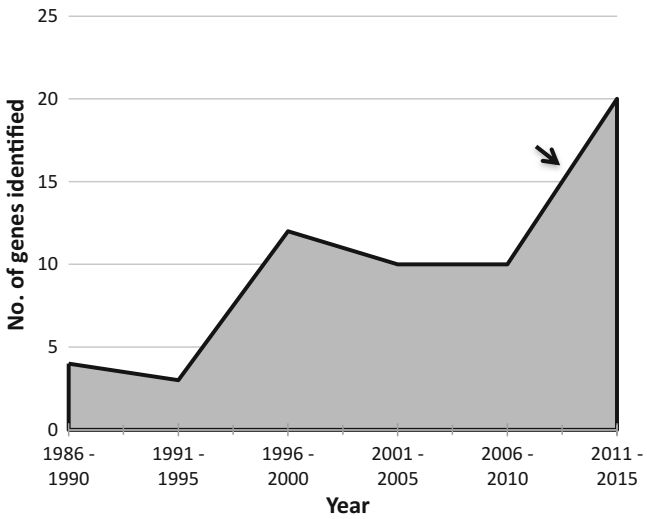

Fig. 12 Rate of gene discovery associated with PFDs. The graph represents the number of genes discovered in association with platelet function disorders in six blocks of 5-year periods between 1986 and 2015. The *arrow* represents the era in which next-generation sequencing technologies have been utilised for the majority of novel genes discovered

Identification of causative variants has obvious importance for diagnosis and in some circumstances can influence the treatment plan (e.g., immune thrombocytopenia versus inherited thrombocytopenia) (Drachman 2004). Greater understanding of the genetic basis could help develop novel treatments for bleeding disorders. Furthermore, increasing our understanding of platelet function may also identify targets for the development of novel antiplatelet agents for the treatment of cardiovascular disease.

Conclusion

The use of NGS technologies has greatly enhanced gene discovery in the field of haemostasis. Target gene panels and WES have been the major focus in the last 5 years, identifying 17 novel genetic associations with platelet bleeding disorders, mainly thrombocytopenia. As WGS becomes increasingly more economical, it is likely that its use will surpass the use of WES and targeted gene panels. Combining the genome data with detailed phenotypic information can provide a further method for candidate gene prioritisation.

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Take Home Message

How has next-generation sequencing (NGS) impacted on the diagnosis of platelet disorders?

- NGS permits large-scale, simultaneous genetic analysis, increasing the accuracy and rate of identification of novel genes and mutations associated with disease.
- Identification of novel genes associated with platelet disorders enhances our understanding of platelet function, which is important in the development of new therapies for the treatment of thrombotic and non-thrombotic disorders.
- As NGS continues to become more cost-effective, the future of diagnosis of platelet disorders is likely to utilise the power of NGS in combination with phenotypic information.

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Part III

Pathology: Hemostatic Disorders

Approach to the Patient with Platelet-Related Bleeding

Diego Mezzano and Jaime Pereira

Abstract

Mucous and skin bleeding are the major symptoms of platelet function disorders (PFDs) and thrombocytopenias. Usually, the severity of bleeding is inversely proportional to the platelet count and may be present in newborns with the more severe platelet aggregation (Glanzmann's disease) and adhesion (Bernard-Soulier (B-S) syndrome) defects. The pattern of bleeding is not characteristic of PFD, since symptoms are similar to those of patients with von Willebrand disease (VWD). Isolated or combined bleeding symptoms are frequent in otherwise healthy population, and bleeding assessment tools (BATs) have been developed to differentiate pathologic bleeding from bleeding proportional to injury. Apart from the non-specificity of symptoms, another confounder is the substantial proportion of clinically similar abnormal bleeders who, after being subjected to a complete battery of laboratory tests, end up without a known diagnosis. An additional difficulty in approaching bleeding and assigning the cause to some suspected PFD is the great variability of tests used in the diagnosis, some of them not fully validated. Furthermore, in the majority of inherited PFD bleeding tends to decrease with age. This chapter will review the clinical approach to bleeding, mainly related to PFD, highlighting the widely dissimilar results of different studies.

Introduction

Platelet function disorders (PFDs), platelet number defects, and also the platelet adhesion defect caused by low von Willebrand factor (VWF) in von Willebrand disease (VWD) are typically classified as disorders of primary hemostasis, expressed with mucous and skin bleeding. In contrast, hemarthrosis and deep muscle bleeding are distinctive symptoms of disorders of secondary hemostasis, as is the case of severe hemophilias. However, the symptoms of patients with mild to moderate deficiencies of clotting factors, including hemophilias, may be difficult to differentiate clinically from bleeding caused by PFD. In such cases, subtle differences may orient the clinician to distinguish primary from secondary hemostatic defects: spontaneous bleeding, immediate surgical bleeding, and petechiae are typical of platelet disorders and thrombocytopenias, whereas bleeding in mild disorders of secondary hemostasis, hyperfibrinolysis, and FXIII deficiency are better characterized by delayed surgical bleeding and are associated with some kind of trauma and petechial bleeding is absent or exceptional. (Table 1 summarizes these differences). An additional difficulty in assessing the cause of primary hemostasis bleeding is presented by the significant fraction of patients with unequivocal pathologic bleeding who end up with no diagnosis after comprehensive and repeated laboratory studies (Quiroga et al. 2007).

The severity of bleeding in PFD is widely variable, depending on the nature of the disorder, but also in not well understood inherited or acquired conditions of the

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Table 1 Bleeding pattern in patients with primary hemostasis disorders (PHD) and patients with secondary hemostasis diseases (SHD) of different severity

	Hemostatic disorders		
Bleeding type	PHD	SHD (mild to moderate deficiency)	SHD (severe)
Petechiae	Yes	No	No
Hemarthrosis, deep hematomas	No	Occasionally, provoked	Yes, spontaneous
Ecchymoses	Yes	Yes	Yes
Mucous bleeding	Spontaneous	Yes, post-trauma	Yes
Surgical bleeding	Yes, immediate	Yes, delayed	Yes, delayed

patient. Generally, those patients with primary aggregation, e.g., Glanzmann's thrombasthenia (GT), or adhesion, e.g., Bernard-Soulier (B-S) syndrome defects, have more severe bleeding than the patients with other congenital (e.g., Hermansky-Pudlak) or secondary (e.g., drug-induced) secretion defects. GT or B-S heterozygous subjects usually have no pathological bleeding, although autosomal dominant negative GT variants exist, most of them associated with a bleeding diathesis of variable severity (Gresele et al. 2009; Nurden et al. 2011). Bleeding in isolated thrombocytopenias is more common when platelet counts fall below 20–30 $\times$ 10^3 platelets μL^{-1} or higher in syndromic inherited thrombocytopenias accompanied with platelet dysfunction; also, bleeding may manifest with higher platelet counts if associated with acquired conditions, such as infections, trauma, and drug intake, among others. It is not uncommon that relatives of index cases with inherited mild PFD share the laboratory abnormality but are clinically asymptomatic. This is analogous with the absence of hemorrhages in the overwhelming majority of subjects on aspirin treatment with a laboratory defect which is usually more severe than that of bleeders with mild inherited PFD.

This chapter will focus on the approach to the bleeding history and physical examination of patients with mucous and skin bleeding, characteristic of primary hemostasis disorders, with special emphasis in platelet disorders.

Bleeding History

A personal bleeding history is probably the most important predictor of bleeding risk. This highlights the importance of a comprehensive evaluation of symptoms and other clinical features, which help discerning between bleeding proportional to trauma and pathological bleeding. The personal and family history may disclose whether a bleeding disorder is inherited or acquired. Also, it must be taken into consideration that other concurrent inherited or acquired conditions or medications could explain or exacerbate bleeding in some patients. Repeated bleeding from several sites is characteristic of hemorrhagic disorders, whereas localized, single site of bleeding orients to a local cause. An overwhelming majority of isolated digestive tract (GI) bleeding, hematuria, or hemoptysis are explained by local causes and not by a genuine bleeding disorder.

A major confounder in differentiating normal from pathological bleeding is the high prevalence of bleeding symptoms among healthy subjects (Quiroga and Mezzano 2012). A report collecting the results of ten different studies in healthy subjects of both genres including children and adults calculated mean frequencies of 23 %, 20 %, 28 %, and 35 % for epistaxis, easy bruising, gum bleeding, and menorrhagia, respectively (Mauer et al. 2011). Another study in more than 1000 young, healthy women found that 73 % had one bleeding symptom, 43 % had two symptoms, and 23 % had more than two symptoms (Friberg et al. 2006). In 299 young healthy subjects "self-classified" as non-bleeders, we estimated that 19 %, 25 %, and 12.7 % had abnormal epistaxis, ecchymoses, and gum bleeding, respectively (Quiroga et al. 2007).

It is easier to qualify the clinical relevance of a bleeding episode in adults who have suffered different hemostatic insults along their lives. Similarly, in severe platelet defects, such as GT, B-S, and some inherited thrombocytopenias, bleeding is frequently evident at delivery, and, furthermore, bleeding may be diagnosed even in fetus suffering from isoimmune thrombocytopenia. In contrast, a genuine mild bleeding disorder may be missed in toddlers and children not yet exposed to hemostatic challenges; and menorrhagia and postpartum bleeding, major symptoms in women with platelet dysfunctions, are only evident after menarche. Finally, since mucous and skin bleeding tend to decrease with age in these patients, some adults do not appraise accurately the magnitude of their early life symptoms and may mislead the physician's perception on the severity of bleeding.

Worldwide, mild bleeding disorders are more frequently diagnosed in women than in men (Sramek et al. 1995; Quiroga et al. 2007; Podda et al. 2007), except for hemophilias. Although the causes for this predominance in women are not fully explained, menorrhagia and postpartum bleeding contribute to it; also, easy bruising is more frequent in women, who are also more aware of this symptom.

Several inherited diseases are characterized by PFD and/or changes in blood platelet count and size. In some of these syndromic defects, clinical bleeding may be only a minor component of the whole syndrome, but these associations facilitate the diagnosis. In this context, alpha-granule defects may be associated with myelofibrosis (gray platelet syndrome); increased fibrinolytic activity (Quebec platelet

disorder); arthrogryposis, renal failure, and cholestasis (gray platelet syndrome-like defect); albinism, immune and neurological disorders, lymphohistiocytosis, and giant inclusion bodies in blood smears (Hermansky-Pudlak, Chédiak-Higashi, and Griscelli syndromes); eczema, immune deficiency, and small platelet size (Wiskott-Aldrich syndrome); thrombocytopenia, giant platelets, giant inclusion bodies in leukocytes, hearing loss, cataracts, renal dysfunction (MYH9-related diseases, such as May-Hegglin anomaly and Sebastian, Epstein, and Fechtner syndromes), osteopetrosis, and thrombocytopenia (Ghosal syndrome); and several inherited thrombocytopenias, with or without abnormal platelet volume, which are associated with diverse bone or organ malformations (Nurden and Nurden 2011; Noris et al. 2014a).

The cause of bleeding in patients with severe acquired thrombocytopenia usually entails few diagnostic problems for the physician. This is the case, among others, of ITP, aplastic anemia, leukemias, and myelosuppressive therapy. More challenging is to diagnose a drug-induced immune thrombocytopenia (ITP), particularly when it is important to identify the causative drug (George and Aster 2009).

Bleeding Scores

In an attempt to overcome the difficulties for assessing the clinical significance of bleeding and to improve diagnostic accuracy, several bleeding scores (BS) have been developed in the last years in the form of structured questionnaires (bleeding assessment tools, BATs), which evaluate semiquantitatively each possible bleeding symptom. The weighted answers of the patients (i.e., site of bleeding, frequency, magnitude, type, need for treatment, and response to it) allow more accurate assessment of the clinical relevance of bleeding. The sum of this record allows adopting a cutoff value useful to distinguish normal from pathological bleeding and, at the same time, assessing its severity.

Of note, one of the first BATs (Sramek et al. 1995) was especially useful in a screening setting in busy clinics to predict future bleeding. The authors found that the history of bleeding following trauma, bleeding from surgery, and a family bleeding disorder were the most informative predictors for future events.

Several BATs have been developed since then (Rydz and James 2012), including the Vicenza BAT (Rodeghiero et al. 2005), the Molecular and Clinical Markers for the Diagnosis and Management of type 1 VWD (MCMDM-1) Bleeding Questionnaire (Tosetto et al. 2006), the Pediatric Bleeding Questionnaire (PBQ) (Bowman et al. 2009), and a bleeding questionnaire developed specifically for the Quebec Platelet Disorder (McKay et al. 2004). In a recent, prospective study in patients with VWD, a MCMDM-1 VWD with a BS >10 predicted the future use of desmopressin or factor concentrate replacement independent of VWD type or VWF levels (Federici et al. 2014). All this moved the International Society on Thrombosis and Haemostasis/Scientific Sub-committee (ISTH-SSC) to adopt and stimulate the use of a BAT ISTH-SSC in 2010, summarizing previous efforts and extending its scope to include bleeding assessment of patients with inherited bleeding diseases other than VWD (Rodeghiero et al. 2010). With regard to PFD, a small preliminary study found that a positive BS identified those patients with PFDs and VWD, whereas a negative BS identified those not needing specific laboratory testing (James et al. 2011); more recently, however, another report showed that ISTH-BAT did not predict the presence of a platelet defect in patients with inherited PFD diagnosed by lumiaggregometry (Lowe et al. 2013). However, confirmation of these initial results is needed to establish the usefulness of BATs in patients with PFD. Currently, consensus exists that BATs are useful tools to identify patients with abnormal bleeding, to provide more objective information on bleeding severity, and to facilitate communication of research results and, additionally, may be a bleeding predictor; however, BATs are still useless for diagnosis of individual diseases or for guiding the order of specific laboratory tests (Sramek et al. 1995).

Another important confounder is the evidence that a high proportion of those patients with unquestionable abnormal bleeding, similar to that of PFD and VWD, end up without a definite diagnosis after exhaustive and repeated laboratory testing (Quiroga et al. 2007). This pattern is constant in several prospective reports and ranges from 47 to 69 % (Parkin et al. 1992; Quiroga et al. 2007; Podda et al. 2007; Gupta et al. 2007; Agren et al. 2006; Marcus et al. 2011; Tosetto et al. 2011).

Table 2 shows the frequency of bleeding from different sites and family history in 65 patients with inherited PFD,

Table 2 Site and frequency of bleeding and family history in patients with platelet function defects

Symptoms	Platelet function defects (%) (*n*: 65)	Controls (%) (*n*: 299)
Menorrhagia	92 (25)[a]	0.3
Ecchymoses	68	19
Epistaxis	68	25
Cauterization	22	1
Tooth extraction bleeding	83 (18)	0
Gum bleeding	48	13
Postpartum hemorrhage	43 (7)	0
Bleeding from minor cuts	50	1
Surgical bleeding	38 (16)	0
Aspirin	31 (16)	0
Family history	88	51

[a]Denotes subjects exposed to the risk

Modified from the original Table 3 in Quiroga T et al. High prevalence of bleeders of unknown cause among patients with inherited mucocutaneous bleeding. A prospective study of 280 patients and 299 controls. Haematologica 2007; 92:357–65, with permission from Haematologica Journal website http://www.haematologica.org

corresponding to 23 % of 280 patients with unequivocal abnormal bleeding. Among these patients, 46 had primary secretion defects (42 with abnormal and four with normal platelet aggregation), and 18 had only defective aggregation. Three of the patients with secretion defects had δ-storage pool disease (low platelet 5-HT and ADP and high ATP/ADP ratio), and the last patient had the phenotypic pattern of GT (Quiroga et al. 2007).

Epistaxis

Nosebleed is one of the most common manifestations of platelet function defects, thrombocytopenias, VWD, and hereditary hemorrhagic telangiectasias (Sandoval et al. 2002). Since a large fraction of the normal population has experienced one or more nosebleeds, it is important to inquire about the frequency, whether it is spontaneous or associated to trauma and if it requires medical evaluation or treatment, including packing and cautery. Bleeding restricted to only one nostril is likely caused by a local vascular abnormality rather than a true hemostatic disorder. It is important to consider the age when evaluating epistaxis, since like other hemorrhages in primary hemostasis disorders, spontaneous nosebleeds tend to diminish or even disappear after puberty.

Purpura

The assessment of clinical significance of bruises (ecchymoses) is difficult, because the recognition of the symptom and perception of its severity vary widely among patients. For example, someone with frequent bruises secondary to minor trauma may consider that this is normal. There is also a clear gender difference, being easy bruising much more common in women than in men. Ecchymoses presenting without noticeable trauma, especially on the trunk or back, and particularly if they are large (>2 in. in diameter), should be judged as pathological. The sudden appearance of new and multiple ecchymoses may be secondary to an acquired condition such as thrombocytopenia or blood coagulation factor inhibitor.

Petechiae typically appear as pinpoint hemorrhages in dependent portions of the vasculature, which do not blanch with pressure. Petechial purpura is characteristic in patients with very low platelet counts and severe platelet defects, such as GT and B-S syndrome. In physiological conditions, platelets maintain the stability of the vasculature through several mechanisms, including constitutive expression of trophogens on the platelet surface or constitutive release of cytokines and growth factors that preserve the structural and functional integrity of the vasculature (Nachman and Rafii 2008). Under a critical number of platelets, these trophic effects on the endothelium are impaired, and the cadherin complex of the vascular endothelium breaks down, allowing extravasation of red cells into the tissues (petechiae). Capillary hemorrhages occur more frequently in areas of increased capillary pressure, such as ankles. In infants, the increase in venous pressure with crying may be enough for the development of petechial lesions. Spontaneous petechiae or hemorrhagic bullae in the oral mucosa usually are a sign of severe thrombocytopenia.

Oral Mucosal Bleeding

The clinical evaluation of gum bleeding is particularly difficult since it is common in apparently healthy subjects, with around 50 % of them expressing this symptom at least occasionally. It is recommended to examine the patients for gum inflammation, which may cause or augment bleeding. In this setting, gum bleeding is a frequent manifestation of PFD, mainly after dental brushing and gum intervention procedures. For these reasons, it is important to record the frequency of gingival hemorrhage and whether it is spontaneous or after trauma or brisk tooth cleaning. Moreover, gingival bleeding is often the first hemorrhagic symptom denoting hemostatic derangement in thrombocytopenias secondary to chemotherapy. Dental extraction, especially of molars, constitutes a major hemostatic challenge, and excessive bleeding after such procedures is highly common in patients with PFD. Moreover, the high fibrinolytic activity of the oral mucosa apparently magnifies the hemostatic impairment, since hemorrhages are commonly controlled with the oral use of antifibrinolytic agents. It is important to assess the magnitude of the blood loss, the duration of hemorrhage (usually several hours), and the need to return to the dentist for packing or suturing.

Bleeding from Minor Cuts

Patients with platelet disorders very often bleed excessively from minor cuts, like shaving. It may be difficult to obtain accurate information regarding bleeding due to razor incisions, although it is of help to ask whether the patient has to delay leaving home because of persistent oozing (Coller and Schneiderman 1995). It is helpful to know if patients change from razor blades to electric razor to avoid bleeding. Bleeding secondary to other minor cuts is often hard to assess unless the patient spontaneously claims that he or she bleeds longer compared with friends or classmates. Some patients also confess longer bleeding when taking aspirin. It is not uncommon that patients perceive that they do not bleed longer than normal but do have a delayed wound scarring and cure (personal observation).

Surgical Bleeding

Details of every surgical procedure must be investigated, including statements made by the surgeon regarding abnormal bleeding during or after surgery. Often, surgeons notice the abnormal bleeding since the skin incision in patients with PFD, denoting oozing from capillaries and small vessels, in contrast with the bleeding observed in mild or moderate clotting factor deficiencies, which is more delayed or occurs after the end of the procedure. Particularly important is to inquire whether a blood transfusion was necessary. Tonsillectomy and adenoidectomy are considered major hemostatic challenges, and the hemorrhage may occur early after surgery or even after the scar falls. In patients undergoing adenotonsillar procedures, it is useful to identify the existence of PFD or other mild bleeding disorders, because adequate management may reduce considerably the hemorrhagic complications (Garcia-Matte et al. 2012). In this regard, hospital records may provide key information regarding clinical and laboratory data ignored by the patient.

Menorrhagia and Postpartum Hemorrhage

Heavy menstrual bleeding or menorrhagia is usually diagnosed when normal menstrual periods last more than 7 days and/or involve blood loss greater than 80 mL. The prevalence of menorrhagia is estimated at 11–13 % in the general population, increasing in women between 36 and 40 years to reach up to 24 % (Marret et al. 2010), and in approximately 50 % of the patients, the etiology remains unexplained. Given that the patient perception of the amount of blood loss during periods is highly subjective and has poor correlation with the actual blood loss, the time length of the menstrual periods is preferred to diagnose heavier than normal blood losses. Other accurate indices to assess the severity of menorrhagia include secondary iron deficiency anemia, the use of contraceptives to manage bleeding, impaired daily activities, and need for emergency hysterectomy or blood transfusions. Some estimations range between 15 % (Oehler and Rees 2003) and 20 % (James et al. 2006) the proportion of women seeking medical attention with an underlying bleeding disorder (Rydz and James 2012). Most of the reports on the etiology have been focused in VWD (James et al. 2009), with a prevalence widely dispersed between 5 and 36 % (Mills et al. 2014). The studies that have addressed the prevalence of PFD have used different diagnostic criteria and laboratory methods (whole blood and platelet-rich plasma lumiaggregometry, platelet function assay [PFA-100™], platelet aggregation with ristocetin). This explains the disparate reports about defects of platelet function, which range between 2 and 56 % in postmenarchial women (Mills et al. 2014; Miller et al. 2011). Table 2 shows that in a population of 25 women with diagnoses of platelet aggregation/secretion defects, measured with light transmission platelet aggregometry and ^{14}C-serotonin release assay, the most frequent symptom was heavy menstrual bleeding, which was present in 23 (92 %) of them (Quiroga et al. 2007).

Menorrhagia is also a common symptom in patients with aplastic anemia as well as in inherited, immune, and drug-induced thrombocytopenias (including post-chemotherapy) (Mathias et al. 2008; Bates et al. 2011).

Thrombocytopenia affects between 5 and 8 % of pregnancies, is likely dilutional in etiology, exceptionally falls below 80×10^3 platelets μL^{-1} (Lefkou and Hunt 2008), and does not cause bleeding. Pregnancies coursing with immune thrombocytopenic purpura have low frequency and are rarely fatal (British Committee for Standards in Haematology General Haematology Task Force 2003). Petechial purpura may be apparent with very low platelet counts, and intra- and postpartum bleeding is uncommon with platelet counts $>50 \times 10^3$ platelets μL^{-1}. Neonatal thrombocytopenia in ITP is severe in around 4 % of the cases (below 20×10^3 platelets μL^{-1}) presenting high risk of hemorrhage at birth. Up to 50 % of the patients with GT have severe bleeds at delivery and require strict control and prophylaxis. Other, less severe PFDs do not have such an increased bleeding risk, but peri-delivery care is recommended, mainly in those women highly symptomatic before pregnancy (Civaschi et al. 2015). Similarly, women with symptomatic hereditary thrombocytopenias and platelet counts below 50×10^3 platelets μL^{-1} have higher risk of bleeding (Noris et al. 2014b).

Central Nervous System Bleeding

Spontaneous hemorrhages in central nervous system are exceptional in patients with inherited PFD, even in those with severe bleeding from other sites (e.g., GT). In contrast, diffuse intracerebral petechial lesions or hemorrhagic strokes may occur in patients with severe thrombocytopenias. In immune thrombocytopenias (ITP), a recent systematic review compiling 51 prospective studies in children and adults showed that the weighted incidences of adult and children intracerebral hemorrhages were 1.4 % and 0.4 %, respectively (Neunert et al. 2015), mainly in those patients with chronic ITP (Neunert et al. 2015). Predictors of parenchymal bleeding were severe thrombocytopenia (<10 or 20×10^3 platelets μL^{-1}), newly diagnosed ITP, and previous minor bleedings (Buchanan and Adix 2002; Rosthoj et al. 2012).

Other Mucosal Bleeding

Hemoptysis is not a presenting symptom of a bleeding disorder, and it is also rare even in patients with severe bleeding diathesis. However, patients with an inherited bleeding disorder might have blood-tingled sputum, especially associated with respiratory tract infections (Quiroga et al. 2007).

GI bleeding is also uncommon as presenting symptom of a bleeding disorder, although a hemostatic defect may trigger or exacerbate a previous GI lesion. In fact, in the series of Quiroga T et al., close to 10 % of the patients with a mild bleeding disorder manifested suffering from previous GI hemorrhage (Quiroga et al. 2007).

Platelet disorders rarely present with hematuria. However, platelet dysfunction and thrombocytopenias may contribute to exacerbate hematuria caused by other disorders, such urinary tract infections. Among patients with VWD and PFD, less than 4 % complained of hematuria (Quiroga et al. 2007). However, hemoptysis, GI bleeding, and hematuria are not uncommon in patients with severe thrombocytopenias of any etiology.

Other rare platelet dysfunctions that must be taken into account are the Quebec platelet disorder (QPD) and the Scott syndrome. Both have a different bleeding pattern when compared with the most frequent PFD. Their key clinical feature is delayed-onset bleeding following surgery, dental procedures, or trauma, but spontaneous bleeding is infrequent. The type of bleeding in patients with QPD is similar to that observed in patients with hyperfibrinolysis and is explained by increased platelet urokinase (uPA) storage, which induces plasmin-mediated digestion of alpha-granules and also platelet release of uPA, which accelerates the clot lysis. The increased plasma fibrin/fibrinogen degradation products and the bleeding response to fibrinolytic inhibitors constitute distinctive features of this disorder (Blavignac et al. 2011). Scott syndrome exhibits a similar bleeding pattern, but in this case a defect of platelet procoagulant activity explains the symptoms. A diminished prothrombin consumption test and a striking decrease in annexin V binding constitute initial diagnostic approaches before genetic confirmation of the molecular defect (Parry et al. 1980; Weiss et al. 1979; Andrews et al. 2002).

Physicians must be alert for suspecting and diagnosing the platelet-type VWD (PT-VWD), a rare platelet function disorder phenotypically similar to type 2B VWD, mainly because both disorders have different treatments. It has been estimated that PT-VWD constitutes up to 15 % of the total number of patients diagnosed with type 2B VWD. Functional assays allow a correct phenotypic diagnosis, before confirmation through genetic testing (Kaur et al. 2014; Maurer et al. 2015).

Hereditary hemorrhagic telangiectasia (disease of Osler-Weber-Rendu) is a developmental disorder of the vasculature, which presents with mucous and skin telangiectases and A-V malformations frequently affecting the GI tract. Patients with this syndrome suffer from nose and GI bleeding without having quantitative and functional platelet defects (McDonald et al. 2011).

The classic subtype of Ehlers-Danlos syndrome, a disorder caused by deficiency of type V collagen, may present with easy bruising with normal platelet function and number. In this case, the diagnosis requires the presence of skin hyperextensibility, widened atrophic scars, and joint hypermobility as major diagnostic criteria (Vanakker et al. 2015).

Effect of Medications and Supplements

Drug-induced bleeding is caused by several drugs that affect platelet function and which are used with therapeutic intent (i.e., aspirin, thienopyridines). However, more than 100 drugs, foods, and supplements have been reported to inhibit the function of platelets; however, conclusive evidence for their association with clinical bleeding of most of these is lacking (Konkle 2011). Moreover, the clinical use of more than 300 drugs has been reported to induce immune thrombocytopenia, although the level of evidence for their pathogenic role is poor for the vast majority of them (George and Aster 2009).

Nonsteroidal anti-inflammatory drugs (NSAIDs) and aspirin impair platelet function by reversible and irreversible inhibition of platelet cyclooxygenase-1, respectively, and may trigger hemorrhages or even unveil a previously unnoticed platelet qualitative or quantitative defect. In fact, aspirin intake exacerbates bleeding in close to 30 % of the patients with PFD (Table 2) (Quiroga et al. 2007), and the combination of aspirin with warfarin increases two- to threefold the risk of major bleeding in patients with coronary stents (Andreotti et al. 2006). Many reports have addressed the issue of major bleeding in patients treated for depression with SSRIs. However, recent reviews show that the evidence for increased bleeding risk in pregnancy and postpartum hemorrhage (Bruning et al. 2015) and major digestive tract bleeding (Jiang et al. 2015) is still inconclusive.

The personal history of the patient must keep detailed information on the use of medications, considering that many drugs have detrimental effects on platelet count (Aster et al. 2009; Arnold et al. 2013) or function (Cattaneo 2011). The clinician should consider the possibility of an underlying hemostatic defect, even if the bleeding is contemporary to drug use.

Several herbal supplements, some foods, and spices may impair hemostasis, although the evidence for a bleeding risk is lacking for most of them. Fish oils rich in omega-3 fatty acids, polyphenols, and cocoa flavonoids, among others,

have been described to prolong the bleeding time and affect platelet function, but there is not enough evidence that their use is associated with bleeding or bleeding enhancement (McEwen 2014; Ostertag et al. 2010; Heptinstall et al. 2006; Srivastava et al. 1995).

Take Home Messages

- Mucous and skin bleeding are the hallmarks of platelet function disorders and thrombocytopenias, like in other disorders of primary hemostasis, but also frequently in mild to moderate clotting factor deficiencies.
- Most inherited platelet function defects are explained by abnormal granule secretion of yet unknown cause and course with a mild bleeding disorder. The severity of bleeding in thrombocytopenias is related to the magnitude of the platelet fall. Clinical bleeding increases when platelet defects coexist with acquired conditions.
- The screening tests (bleeding time, PFA-100™) lack sensitivity and are non-specific for diagnosing a particular disorder of primary hemostasis, excepting thrombocytopenias and mild/moderate clotting factor deficiencies.
- Initial laboratory diagnosis in patients with conclusive abnormal bleeding and/or high bleeding scores should initially include the study of von Willebrand disease but also of PFD.

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Inherited Thrombocytopenias

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Abstract

Inherited thrombocytopenias are a heterogeneous group of disorders with different degrees of severity and complexity deriving from mutations in at least 25 genes. Only a few inherited thrombocytopenias are characterized by recurrent spontaneous hemorrhages due to a very low platelet count and/or associated platelet dysfunction, while the other forms expose patients to the risk of bleeding in connection with hemostatic challenges. Some of the genetic abnormalities resulting in thrombocytopenia also cause additional congenital defects. Moreover, some common forms of inherited thrombocytopenia predispose to acquire in childhood or adult life additional serious diseases, as bone marrow aplasia, hematological malignancies, or kidney failure. Making a definite diagnosis is difficult and requires a close collaboration of clinicians, lab technicians, and geneticists. Many therapeutic measures are available both for preventing and stopping bleeding. Different treatments are available also for many congenital or acquired defects that may associate with thrombocytopenia.

Introduction

Inherited thrombocytopenias (ITs) include at least 26 disorders caused by mutations in 30 different genes (Table 1), but their number is expected to be higher because a definite diagnosis is not possible in about half of patients as they do not meet the criteria for any of the diseases described so far. Therefore, new ITs are yet to be discovered.

ITs are rare diseases, with an estimated prevalence of at least 2.7 in 100,000 (Balduini et al. 2012). However, this figure probably underestimates their actual frequency because they are often misdiagnosed with immune thrombocytopenia (ITP).

ITs are clinically very heterogeneous, with great variability in the degree of platelet deficiency and bleeding tendency, as well as in the additional defects that may associate with thrombocytopenia. Moreover, some of these additional manifestations are present since birth, while others develop later in life.

Classification

Different types of classification have been proposed. For their usefulness in the diagnostic process, the classification based on the presence of defects that add to thrombocytopenia (Balduini et al. 2013) and the one based on the size of the platelets, which varies greatly from one disorder to another (Noris et al. 2014a), are the most commonly used. Also the classification of ITs according to the mode of transmission may be useful for diagnostic purposes, but the type of inheritance is not always identifiable because of the frequent occurrence of sporadic cases due to de novo mutations or recessive inheritance.

The classification we use in Table 1 takes into account both the presence of defects added to thrombocytopenia since birth and the risk of acquiring additional defects during

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Table 1 Main features of inherited thrombocytopenias classified according to their clinical picture

Disease (abbreviation, OMIM entry) (references)	Reported families[a]	Inheritance	Gene (locus)	Thrombocytopenia[b]	Bleeding[c]	Additional features
Forms with additional clinically relevant congenital defects						
Wiskott–Aldrich syndrome (WAS, 301000) (Massaad et al. 2013)	++++	XL	*WAS* (Xp11)	+++	+++	Severe immunodeficiency. Eczema. Increased risk of malignancies and autoimmunity
X-linked thrombocytopenia (XLT, 313900) (Albert et al. 2010)				++/+++	++/+++	Thrombocytopenia may be intermittent. Mild immunodeficiency. Mild transient eczema. Increased risk of malignancies and autoimmunity. Also non-syndromic[d]
Paris–Trousseau thrombocytopenia (TCPT, 188025), Jacobsen syndrome (JBS, 147791) (Favier et al. 2015)	++++	AD	Deletions in 11q23	+++	++/+++	Thrombocytopenia may resolve over time. Growth delay, mental retardation, facial dysmorphisms, malformations of the cardiovascular system, CNS, gastrointestinal apparatus, kidney, and/or urinary tract; other malformations
Thrombocytopenia-absent radius syndrome (TAR, 274000) (Toriello 2011)	+++	AR	*RBM8A* (1q21)	+++	+++	Platelet count tends to rise over time and often normalizes. Bilateral radial aplasia ± other upper and lower limb bone abnormalities. Reduced/absent megakaryocytes in BM. Possible kidney, cardiac, and/or CNS malformations. Possible intolerance to cow's milk
GATA1-related diseases: X-linked thrombocytopenia with thalassemia (XLTT, 314050), X-linked thrombocytopenia with dyserythropoietic anemia (XLTDA, 300367) (Millikan et al. 2011)	++	XL	*GATA1* (Xp11)	+++	++/+++	Hemolytic anemia with laboratory abnormalities resembling beta-thalassemia, splenomegaly, dyserythropoietic anemia. May present with congenital erythropoietic porphyria
Thrombocytopenia associated with sitosterolemia (STSL, 210250) (Rees et al. 2005)	++	AR	*ABCG5*, *ABCG8* (2p21)	+/++	+/++	Hemolytic anemia with stomatocytosis. Splenomegaly. Early development of tendon and tuberous xanthomas. Premature atherosclerosis. Also non-syndromic[d]
Radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT, 605432) (Albers et al. 2012)	+	AD	*HOXA11* (7p15)	+++	+++	Bilateral radioulnar synostosis ± other malformations. Reduced/absent megakaryocytes in BM. Possible evolution to bone marrow aplasia
FLNA-related thrombocytopenia (*FLNA*-RT, na) (Nurden et al. 2011a, b)	+	XL	*FLNA* (Xq28)	++	++	Periventricular nodular heterotopia (OMIM 300049). Also non-syndromic[d]
THPO-related disease (*THPO*-RD, na) (Dasouki et al. 2013)	+	AR	*THPO* (3q27.1)	++/+++	++	Bone marrow hypoplasia. Red cell macrocytosis. Possible mild thrombocytopenia in heterozygous subjects
Stormorken syndrome (STRMK, 185070) (Misceo et al. 2014)	+	AD	*STIM1* (11p15.4)	+/++	+/+++	Mild anemia, asplenia, myopathy, miosis, headache, ichthyosis, dyslexia
Forms predisposing to other illnesses						
MYH9-related disease (*MYH9*-RD, na) (Balduini et al. 2011a)	++++	AD	*MYH9* (22q12)	+/+++	+/+++	Risk of sensorineural deafness, nephropathy, cataract (genotype–phenotype correlations identified). Elevated liver enzymes in some

(continued)

Table 1 (continued)

Disease (abbreviation, OMIM entry) (references)		Reported families[a]	Inheritance	Gene (locus)	Thrombocytopenia[b]	Bleeding[c]	Additional features
							cases. Döhle-like inclusions in granulocytes
Congenital amegakaryocytic thrombocytopenia (CAMT, 604498) (Ballmaier and Germeshausen 2011)		+++	AR	*MPL* (1p34.2)	+++	+++	Reduced/absent megakaryocytes in BM. Evolution to severe bone marrow aplasia in infancy in all patients
Familial platelet disorder with propensity to acute myelogenous leukemia (FPD-AML, 601399) (Liew and Owen 2011)		++	AD	*RUNX1* (21q22)	++	++	Over 40 % of patients acquire acute myelogenous leukemia or myelodysplastic syndromes. Increased risk of T acute lymphoblastic leukemia
ANKRD26-related thrombocytopenia (*ANKRD26*-RT or THC2, 188000) (Noris et al. 2011)		++	AD	*ANKRD26* (10p12)	++/+++	+/++	About 8 % of patients acquire myeloid malignancies. Some patients have increased levels of hemoglobin and/or leukocytes
ETV6-related thrombocytopenia (*ETV6*-RT, na) (Noetzli et al. 2015)		+	AD	*ETV6* (12p13)	+/++	+	Increased risk of myeloid and lymphoid malignancies
Forms with only thrombocytopenia							
Bernard–Soulier syndrome (BSS, 231200/153670)	Biallelic (Savoia et al. 2014)	++++	AR	*GP1BA* (17p13) *GPIBB* (22q11) *GP9* (3q21)	++/+++	++/+++	
	Monoallelic (Noris et al. 2012)	+++	AD		+	+	
Gray platelet syndrome (GPS, 139090) (Kahr et al. 2011)		++	AR	*NBEAL2* (3p21)	++/+++	+/+++	Platelet count decreases over time. Development of progressive bone marrow fibrosis and splenomegaly. Elevated serum vitamin B12 levels
ACTN1-related thrombocytopenia (*ACTN1*-RT, 615193) (Bottega et al. 2015)		++	AD	*ACTN1* (14q24)	+	+	
Platelet-type von Willebrand disease (PTvWD, 177820) (Othman and Emsley 2014)		++	AD	*GP1BA* (17p13)	+/+++	+/+++	Platelet count is usually normal in most patients, but may decrease greatly under stress conditions (pregnancy, surgery, and infection)
ITGA2B/*ITGB3*-related thrombocytopenia (*ITGA2B*/*ITGB3*-RT, 187800) (Nurden et al. 2011b)		+	AD	*ITGA2B* (17q21), *ITGB3* (17q21)	+/++	+/++	
TUBB1-related thrombocytopenia (*TUBB1*-RT, 613112) (Kunishima et al. 2009)		+	AD	*TUBB1* (20q13)	+	+	
CYCS-related thrombocytopenia (*CYCS*-RT or THC4, 612004) (Morison et al. 2008)		+	AD	*CYCS* (7p15)	+	+	
GFI1b-related thrombocytopenia (*GFI1b*-RT, 187900) (Monteferrario et al. 2014)		+	AD	*GFI1B* (9q34)	+/++	++/+++	Red cell anisocytosis
PRKACG-related thrombocytopenia (*PRKACG*-RT, 616176) (Manchev et al. 2014)		+	AR	*PRKACG* (9q21)	+++	+++	

(continued)

Table 1 (continued)

Disease (abbreviation, OMIM entry) (references)	Reported families[a]	Inheritance	Gene (locus)	Thrombocytopenia[b]	Bleeding[c]	Additional features
FYB-related thrombocytopenia (*FYB*-RT, na) (Levin et al. 2015)	+	AR	*FYB* (5p13.1)	++/+++	+/++	
SLFN14-related thrombocytopenia (*SLFN14*-RT, na) (Fletcher et al. 2015)	+	AD	*SLFN14* (17q12)	+/++	++/+++	
FLI1-related thrombocytopenia (*FLI1*-RT, na) (Stevenson et al. 2015a)	+	AR	*FLI1* (11q24.3)	++	++	

[a]N° of reported families: +, less than 10 reported families; ++, more than 10 reported families; +++, more than 50 reported families; ++++, more than 200 reported families
[b]Degree of thrombocytopenia: +, >100 × 10^9 platelets/L; ++, 50–100 × 10^9 platelets/L; +++, <50 × 10^9 platelets/L
[c]Severity of bleeding tendency: +, absent or mild; ++, moderate; S +++, severe
[d]"Also non-syndromic" indicates syndromic forms for which some patients with only thrombocytopenia (without the associated defects) have been reported
Abbreviations: *AD* autosomal dominant, *AR* autosomal recessive, *XL* X-linked, *na* not available, *CNS* central nervous system, *BM* bone marrow

childhood or the adult life. Thus, this type of classification serves both diagnostic and prognostic purposes.

Clinical Picture

The main characteristics of ITs are reported in Table 1.

Bleeding Tendency and Platelet Count

The risk of bleeding in subjects with more than 100 × 10^9 platelets/L is not significantly different from that of healthy people. Patients with platelet counts between 50 and 100 × 10^9/L usually do not bleed spontaneously, but are at risk of hemorrhages on the occasion of major hemostatic challenges. When platelets are less than 50 × 10^9/L, especially when their count is below 20 × 10^9/L, patients may present with spontaneous mucocutaneous bleeding, such as petechiae, easy bruising, nose bleeds, menorrhagia, and gastrointestinal bleeding. Moreover, any hemostatic challenge may result in profuse blood loss.

Although the severity of thrombocytopenia and that of bleeding diathesis are usually closely related, this does not apply to the ITs deriving from genetic anomalies that also cause a defect of platelet function (Table 2), in that affected subjects have a bleeding tendency that is more severe than expected on the basis of platelet count. The most clinically relevant platelet dysfunction is observed in biallelic Bernard–Soulier syndrome (BSS), where platelets have defective glycoprotein (GP) Ib/IX/V complex and therefore fail to interact with the subendothelium at the site of vascular injuries (Berndt and Andrews 2011; Savoia et al. 2011). In this disorder, the severity of bleeding tendency does not associate at all with the degree of thrombocytopenia, as bleeding mainly derives from platelet dysfunction. Although to a lesser extent, platelet function is affected in gray platelet syndrome (GPS), *GFI1b*-related thrombocytopenia (*GFI1b*-RT), familial platelet disorder with propensity to acute myelogenous leukemia (FPD-AML), Paris–Trousseau thrombocytopenia (TCPT)/Jacobsen syndrome (JBS), *FLI1*-related thrombocytopenia (*FLI1*-RT), *PRKACG*-related thrombocytopenia (*PRKACG*-RT), *ITGA2B*/*ITGB3*-related thrombocytopenia (*ITGA2B*/*ITGB3*-RT), sitosterolemia (STSL), *SLFN14*-related thrombocytopenia (*SLFN14*-RT), and *FLNA*-related thrombocytopenia (*FLNA*-RT).

At variance, bleeding diathesis seems sometimes less severe than expected based on the platelet count. This discrepancy is only apparent, since it derives from the fact that the most commonly used cell counters identify platelets by their size and therefore fail to recognize the large platelets that are typical of many ITs (Noris et al. 2009). The difference between the actual platelet count and that measured by the counter may be very large in *MYH9*-related disease (*MYH9*-RD), biallelic BSS, *PRKACG*-RT, and STSL, where a high proportion of platelets are as large as red cells or even larger. Manual platelet counting with an optical microscope in a counting chamber (England et al. 1984) or cell counters that recognize platelets by the binding of platelet-specific antibodies provide the actual value of platelet concentration.

In most cases, the degree of thrombocytopenia remains stable in each patient during lifetime, but some exceptions are worthy of mention. Platelet count tends to raise over time and often normalizes in thrombocytopenia-absent radius syndrome (TAR) and TCPT/JBS (Toriello 2011), while a transient increase in platelet count may be observed in congenital amegakaryocytic thrombocytopenia (CAMT)

Table 2 Pathogenetic mechanisms of inherited thrombocytopenias and coexistence of clinically relevant defects of platelet function

Disease (references)	Function of defective gene	Pathogenesis of thrombocytopenia (defective platelet function)
Defect in early phases of megakaryopoiesis		
Congenital amegakaryocytic thrombocytopenia (Hirata et al. 2013)	*MPL* encodes for the receptor for thrombopoietin (THPO), a hematopoietic growth factor that regulates multipotent hematopoietic progenitor cells and promotes their differentiation into Mks	THPO defect affects commitment–differentiation of multipotent stem cells to Mk. Acquired bone marrow aplasia is due to failure to maintain multipotent hematopoietic cells
Thrombocytopenia-absent radius syndrome (Albers et al. 2013)	*RBM8A* encodes for the subunit member Y14 of the exon junction complex, which is involved in RNA processing and required for same basic cellular functions as nuclear export and subcellular localization of specific transcripts	It has been hypothesized that the low expression of *RBM8A* affects mRNA processing of unknown components of the THPO/MPL pathway
Radioulnar synostosis with amegakaryocytic thrombocytopenia (Horvat-Switzer and Thompson 2006)	*HOXA11* encodes for a homeobox transcription factor that regulates morphogenesis and differentiation. Its role in hemopoiesis is not clear	The molecular mechanisms translating HOXA11 mutations in amegakaryocytic thrombocytopenia, and, sometimes, bone marrow aplasia are unknown
THPO-related disease (Dasouki et al. 2013)	*THPO* is the gene for thrombopoietin, which is essential for survival of multipotent hematopoietic cells and for their differentiation into Mks	Defective thrombopoietin is expected to have consequences similar to those deriving from defective thrombopoietin receptor
Defect in megakaryocyte maturation		
Familial platelet disorder with propensity to acute myelogenous leukemia (Sakurai et al. 2014)	*RUNX1* encodes for the DNA-binding a subunit of the core binding factor (CBF) transcription complex. The CBF regulates the homeostasis between proliferation and differentiation of stem cells in adult hematopoiesis	Reduced expression of the THPO receptor. Defective Mk differentiation and maturation. Defective proplatelet formation. (Alpha/delta storage pool deficiency. Defect of $\alpha_{IIb}\beta_3$ activation and platelet aggregation)
ANKRD26-related thrombocytopenia (Bluteau et al. 2014)	The role of *ANKRD26* in megakaryopoiesis is poorly defined. The binding of RUNX1 and FLI1 silences *ANKRD26* during the latest phases of normal megakaryopoiesis	Loss of *ANKRD26* silencing induced by RUNX1 and FLI1 during Mk maturation cause persistent THPO/MPL pathway signaling. Dysmegakaryopoiesis and impaired proplatelet formation
ETV6-related thrombocytopenia (Noetzli et al. 2015)	*ETV6* encodes a transcriptional repressor that was initially identified as a tumor suppressor	Defective megakaryocyte maturation
FLI1-related thrombocytopenia (Stevenson et al. 2015a)	*FLI1* promotes platelet biogenesis by transactivation of many genes associated with Mk development, such as *MPL*, *GP9*, *GP1BA*, *ITGA2,* and *PF4*	Defective Mk maturation. (Defect of platelet GPVI, GPIb/IX, GPIIb, and abnormal alpha granules. Defect of platelet aggregation)
Paris–Trousseau thrombocytopenia/ Jacobsen syndrome (Favier et al. 2015)	Contiguous gene deletion syndrome. Different sizes and breakpoints of the deletions are responsible for the heterogeneity of the clinical picture. Deletion of *FLI1* causes thrombocytopenia	Maturation block in the megakaryocytic linage, with abundance of immature, hypolobulated, and dystrophic Mks. (Defect of platelet aggregation)
GATA1-related diseases (Songdej and Rao 2015)	*GATA1* regulates genes expressed in the Mk lineage, as *GP1BA*, *GP1BB*, *PF4*, *MPL,* and *NFE2*, and in the erythroid line, as *HBB*, *ALAS1,* and *BCL2L1*.	Alteration of Mk maturation. (Defective platelet aggregation after ristocetin and collagen in some cases)
GFI1b-related thrombocytopenia (Monteferrario et al. 2014)	GFI1B is a transcriptional factor involved in homeostasis of hematopoietic stem cells and development of the megakaryocytic and erythroid lineages	Altered Mk maturation with mild myelofibrosis and defective expression of several platelet proteins, as fibrinogen, P-selectin, and GPIbα. (Many pale platelets due to α alpha-granule deficiency. Variable defect of platelet activation and aggregation)
Gray platelet syndrome (Pecci and Balduini 2014)	*NBEAL2* codes for neurobeachin-like protein 2, which probably plays a role in protein–protein interactions, membrane dynamics, and vesicle trafficking	Defective Mk maturation. (Severe deficiency of platelet alpha granules causing "pale" platelets. Possible defects of platelet aggregation)
FYB-related thrombocytopenia (Levin et al. 2015)	The *FYB* protein (ADAP) is a candidate linker between cell membrane activation signals and intracellular events that regulate actin polymerization and organization	Because of the findings of immature Mks at bone marrow evaluation and activated platelets in blood, it has been hypothesized that thrombocytopenia derives from defective maturation of Mks and clearance of activated platelets

(continued)

Table 2 (continued)

Disease (references)		Function of defective gene	Pathogenesis of thrombocytopenia (defective platelet function)
Defect in platelet release			
MYH9-related disease (Balduini et al. 2011b)		The *MYH9* protein (NMMHC-IIA) is a motor molecule for cellular processes requiring force. It regulates the cytoskeleton and also acts as an end point for many signaling pathways, representing a master integrator of the activities that drives cell migration, cell–cell interaction, and cell-matrix adhesion	Ectopic platelet release resulting in ineffective platelet production and defective proplatelet formation
ACTN1-related thrombocytopenia (Kunishima et al. 2013)		The *ACTN1* protein (actinin, alpha1) organizes actin filaments into bundles	Defective proplatelet formation
FLNA-related thrombocytopenia (Nurden et al. 2011a)		The *FLNA* protein (filamin A) connects actin filaments to the cellular membrane. In platelets, filamin A tethers GPIbα and $\alpha_{IIb}\beta_3$ to the underlying cytoskeleton	Defective proplatelet formation has been hypothesized
Bernard–Soulier syndrome	Biallelic (Balduini et al. 2011b)	The genes *GP1BA*, *GP1BB,* and *GP9* code for GPIbα, GPIbβ, and GPIX, respectively, which form the membrane GPIb-IX-V complex in platelets and Mks. The intracellular domain of GPIbα binds the cytoskeleton though filamin A, the extracellular domain binds to von Willebrand factor	Defective proplatelet formation. (Severely reduced platelet adhesion to exposed subendothelium)
	Monoallelic (Balduini et al. 2009)		Mild defect of proplatelet formation
ITGA2B/*ITGB3*-related thrombocytopenia (Bury et al. 2012)		The gene *ITGA2B* and *ITGB3* encode the components of the $\alpha_{IIb}\beta_3$ complex, a fibrinogen receptor in Plts and Mks	A defective proplatelet formation has been hypothesized. (Defect of platelet aggregation)
TUBB1-related thrombocytopenia (Kunishima et al. 2009)		The *TUBB1* protein (β1 tubulin) is a major component of microtubules in Plts and Mks	Defective proplatelet formation
CYCS-related thrombocytopenia (Morison et al. 2008)		*CYCS* encodes cytochrome c, a mitochondrial protein that participates in mitochondrial respiration and initiation of the intrinsic pathway of apoptosis	It has been hypothesized that an ectopic, premature proplatelet formation causes thrombocytopenia
PRKACG-related thrombocytopenia (Manchev et al. 2014)		*PRKACG* encodes the gamma isoform of the catalytic subunit of cAMP-dependent protein kinase A	Defective proplatelet formation. (Defective platelet activation)
Shortened platelet survival			
Platelet-type von Willebrand disease (Guerrero et al. 2009)		The gene *GP1BA* encodes GPIbα, whose extracellular domain binds to von Willebrand factor at the site of vascular injuries. Mutations results in gain of function of GPIbα	It has been suggested that the increased affinity of platelet GPIbα for circulating von Willebrand factor results in platelet clumping and shortened platelet survival
Wiskott–Aldrich syndrome (Massaad et al. 2013)		The WAS protein plays a key role as regulator of the actin cytoskeleton	Premature, ectopic release of platelets in the bone marrow and increased clearance of platelets from circulation
X-linked thrombocytopenia (Massaad et al. 2013)			
Thrombocytopenia associated with sitosterolemia (Rees et al. 2005)		*ABCG5* and *ABCG8* proteins regulate plant sterol and cholesterol absorption and elimination from the body	It has been suggested that platelets are a target for the toxic effect of increased plasma phytosterols. (Defective platelet aggregation)
Unknown defect			
SLFN14-related thrombocytopenia (Fletcher et al. 2015)		The role of *SLFN14* protein is poorly known. Possible role as an endoribonuclease, regulating rRNA, and ribosome-associated mRNA cleavage	Pathogenesis of thrombocytopenia is unknown. (Defects of platelet dense granules and defective platelet aggregation)
Stormorken syndrome (Morin et al. 2014)		STIM1 is involved in store-operated Ca^{2+} entry	Gain of function mutations result in thrombocytopenia by an unknown mechanism. Platelets have high Ca^{2+} basal levels and are in a preactivated state

(Ballmaier and Germeshausen 2011). Transient increases in platelet count have been observed also in patients with *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT) on the occasion of severe infections, probably because thrombopoietin (THPO) behaves as an acute phase protein and its increase in inflammatory conditions stimulates platelet production (Noris et al. 2011). Personal experience indicates that this phenomenon may occur also in other ITs. At variance, platelet count typically decreases over time in GPS, as a result of progressive marrow fibrosis and splenomegaly (Gunay-Aygun et al. 2010).

Other Congenital Defects

Many of the genetic abnormalities that are responsible for thrombocytopenia cause additional congenital defects (Fig. 1): bone malformations characterize TAR and radioulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT) (Thompson et al. 2001); growth delay, mental retardation, facial dismorphisms, and a variety of other congenital malformations affect subjects with TCPT/JBS (Mattina et al. 2009); the complex neurological defects typical of periventricular nodular heterotopia are present in most cases of *FLNA*-related thrombocytopenia (*FLNA*-RT) (Nurden et al. 2011a). Hemolytic and dyserythropoietic anemia associate with thrombocytopenia in X-linked thrombocytopenia with thalassemia (XLTT) and X-linked thrombocytopenia with dyserythropoietic anemia (XLTDA) (Millikan et al. 2011), respectively, while bone marrow failure of variable severity has been described in *THPO*-related disease (*THPO*-RD) (Dasouki et al. 2013). Finally, patients with Wiskott–Aldrich syndrome (WAS) have severe immune dysregulation, resulting in recurrent infections, eczema, autoimmune phenomena, and increased risk of malignancies, especially lymphoproliferative disorders (Massaad et al. 2013).

Predisposition to Acquire Additional Illnesses

A recently identified aspect of ITs is that some of them predispose to acquire in childhood or adult life specific disorders that may endanger the life of patients much more than thrombocytopenia itself. The most complex and better characterized of these forms is *MYH9*-RD, which predisposes not only to presenile cataracts and sensorineural deafness but also to a proteinuric nephropathy that occurs at a mean age of 27 years and often evolves into end-stage renal failure (Seri et al. 2003). Proteinuria affects 25 % of patients, and about 40 % of them develop end-stage renal disease. Reliable genotype–phenotype correlations have been identified, with some mutations resulting in nephropathy in nearly all cases and others associated with no risk at all (Fig. 2). Similar results have been obtained for cataracts and deafness (Pecci et al. 2014a).

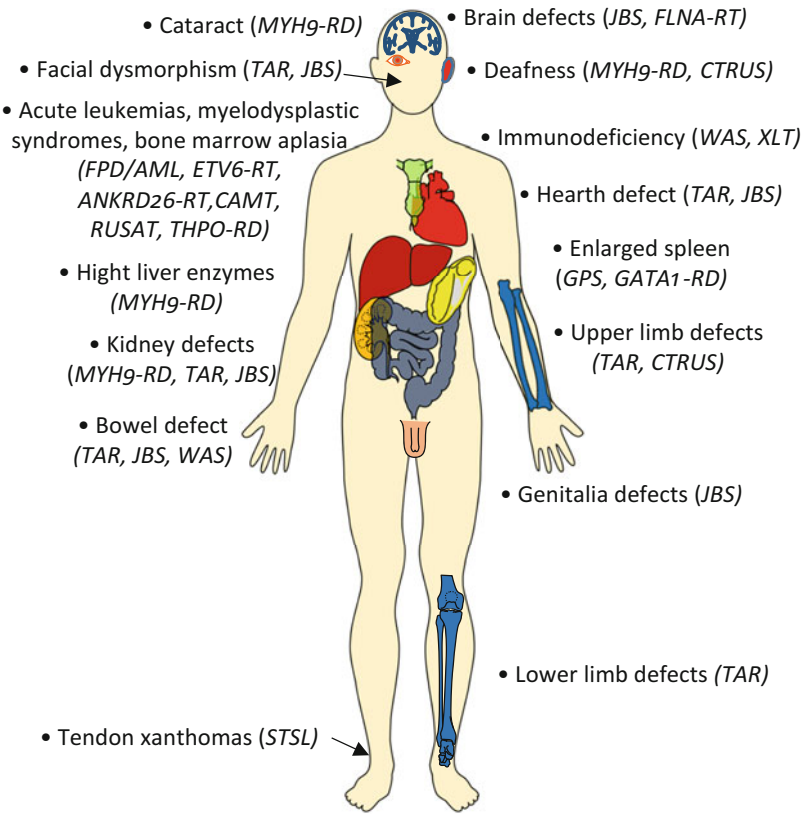

Fig. 1 Defects associated with low platelet count in some inherited thrombocytopenias. Schematic view of the most important defects/diseases, either congenital or acquired, that may affect patients with inherited thrombocytopenias. For abbreviations of disorders, see Table 1

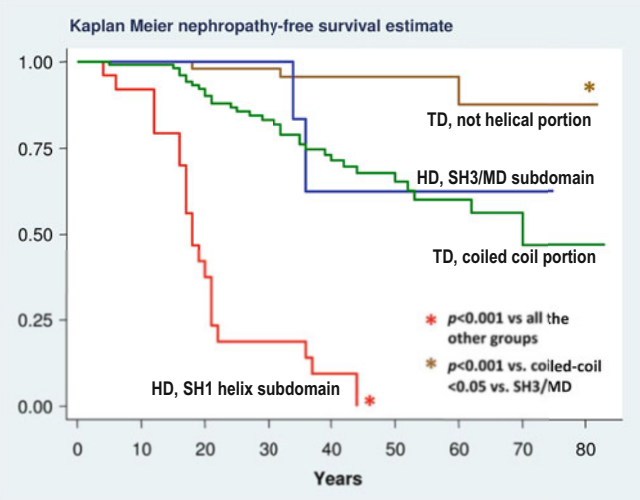

Fig. 2 Genotype–phenotype correlations in 255 patients with *MYH9*-related disease. MYH9 protein comprises three distinct domains: the N-terminal globular head domain (HD), which includes the SH1 helix and the SH3/MD subdomains; the neck; the C-terminal tail domain (TD), which includes the coiled coil portion and the C-terminal not helical portion. Propensity to develop nephropathy varies greatly depending on the location of the causative mutations. All subjects with mutations in the SH1 subdomain of HD (*red line*) are expected to develop nephropathy before age 45, while those with mutations in the terminal, not helical portion of the TD (*brown line*), very rarely suffer from this defect. Patients with mutations in the coiled-coil portion of TD (*green line*) or the SH3/MD subdomain of HD (*blue line*) have intermediate risk, and about 50 % of them develop kidney defect (modified from Pecci et al. 2014a, with permission)

Other forms of ITs are characterized by the propensity to develop hematological malignancies. Over 40 % of subjects with FDP-AML acquire acute myelogenous leukemia or myelodysplastic syndromes, with a median age at onset of 33 years (Liew and Owen 2011). Also the risk of acute lymphoblastic leukemia seems to be increased. The incidence of acute myeloid leukemia and that of chronic myelogenous leukemia is more than 20 times higher in *ANKRD26*-RT than in general population, while that of myelodysplastic syndrome is 10 times higher (Noris et al. 2011, 2013). Finally, several cases of acute lymphoblastic leukemia and other hematological malignancies have been reported in *ETV6*-related thrombocytopenia (*ETV6*-RT) (Noetzli et al. 2015; Zhang et al. 2015).

The most fearsome form of IT in terms of evolution is CAMT, in that it progresses in all cases to a bone marrow aplasia that is fatal unless patients receive hematopoietic stem cell transplantation (Ballmaier and Germeshausen 2011).

Pathogenesis

Defective platelet production is the cause of thrombocytopenia in most ITs, whereas in only few forms the low platelet count derives from reduced survival of platelets. Table 2 succinctly describes the pathogenesis of each IT and the functional role of the involved genes. It also indicates whether a defect of platelet function associates with thrombocytopenia. For a detailed discussion of the pathogenetic mechanisms of ITs, we refer to a recent review dedicated to this topic (Pecci and Balduini 2014).

A small group of ITs is characterized by the absence or a severely reduced amount of megakaryocytes (Mks) in the bone marrow due to defective commitment–differentiation of multipotent stem cells to the Mk lineage. Defects of THPO, its receptor (MPL), or the signaling pathways stimulated by the binding of THPO to MPL have been hypothesized or proven as the causes of these disorders. Of note, while CAMT always evolves into bone marrow aplasia, different degrees of bone marrow hypoplasia may be observed in RUSAT and *THPO*-RD. This indicates that the THPO-MPL pathway is important not only for megakaryocytic differentiation but also for maintenance of the multipotent hematopoietic progenitor compartment.

Defective Mk maturation is the main pathogenetic mechanism of thrombocytopenia in many ITs, although in some forms other mechanisms, as shortened platelet survival or impaired proplatelet formation, contribute to the low platelet count. The genes affected in these ITs are in most cases transcription factors responsible for the fine-tuning of activation and inactivation of specific genes that must be expressed at some stages of megakaryocytic maturation and silenced in others phases. Three of these forms (FPD/AML, *ANKRD26*-RT, and *ETV6*-RT) are characterized by dysmegakaryopoiesis and predisposition to hematological malignancies. Based on this observation, these ITs can be considered as inherited forms of the unilineage myelodysplastic syndrome affecting the Mk lineage (refractory thrombocytopenia) (Gyan et al. 2015).

In another large group of ITs, platelet deficiency derives from a defect in the complex mechanisms that allow mature Mks to release platelets into the lumen of bone marrow sinusoids. In most cases, mature Mks fail to extend properly the long cytoplasmic protrusions from which platelets detach. Less frequently, a premature and/or ectopic release of platelets within the bone marrow results in ineffective thrombopoiesis. ITs deriving from altered platelet release are mainly caused by mutations in cytoskeleton components or membrane integrins.

Finally, shortened platelet survival is the main cause of thrombocytopenia in three ITs. In WAS/XLT, platelets are intrinsically fragile and are prematurely destroyed by the spleen. This is the reason why splenectomy increases or even normalizes platelet count in this condition (see below "Therapy"). In platelet-type von Willebrand disease (PT-VWD), the affinity of platelet GPIbα for the von Willebrand factor (VWF) is increased, and platelets bind spontaneously large circulating multimers of this molecule. It has been suggested that the removal from circulation of

platelet-VWF complexes is the cause of thrombocytopenia, but that there is no experimental evidence of this in humans. At variance, platelets are intrinsically normal in thrombocytopenia associated with STSL, but the high blood levels of phytosterols that characterize this disorder have a toxic effect on their membrane and shorten their survival.

Diagnosis

Two major difficulties are encountered in the diagnostic process of ITs: recognition of patients with a genetic form among individuals with thrombocytopenia and identification of the specific disorder of each patient.

Recognize that Thrombocytopenia Has a Genetic Cause

As a matter of fact, a large proportion of patients are diagnosed as affected by an IT only after a previous misdiagnosis of ITP. The risk of misdiagnosis is well illustrated by the finding that 57 of 181 consecutive women with ITs had a previous diagnosis of ITP (Noris et al. 2014b). For this reason, 44 of them had received treatments that are not only useless, but even dangerous, such as immunosuppressive agents and splenectomy. In many IT patients, the low platelet count is found incidentally in adult life, and the genetic origin of thrombocytopenia is frequently overlooked (Balduini et al. 2013). To avoid this oversight, it is recommended to consider the possibility that every unexplained thrombocytopenia has a genetic origin whenever it is not possible to ascertain that platelet count was normal in the past. In this case, medical history, physical examination, and microscopy examination of peripheral blood films are crucial to identify symptoms and signs that are suggestive of ITs.

Medical History

The finding that other family members of the proband have low platelet counts clearly supports the hypothesis of an IT. However, a negative family history does not exclude this possibility, because some ITs are transmitted in a recessive manner or derive from de novo mutations. For instance, near 40 % of probands with *MYH9*-RD have sporadic forms arising from de novo mutational events (Balduini et al 2011a). Also the presence of bleeding tendency since birth or on the occasion of the first hemostatic challenges, as trauma, menarche, or surgery, supports the hypothesis of a genetic defect. Another finding suggestive for ITs is bleeding tendency more severe than expected based on platelet count, since defective platelet function characterizes many IT forms (Table 2).

As some ITs are associated with other congenital defects or predispose to other illnesses, medical history should not be limited to bleeding diathesis; moreover, the search for associated manifestations should be performed not only in the proband but in all the family members. Indeed, the associated defects may have variable expressivity within the same families and/or may occur late in life. In the latter case, they may be absent in young individuals but present in older affected relatives. For instance, young patients with *MYH9*-RD usually do not present with kidney damage, deafness, and/or cataracts, which are instead frequently found in older family members with this disorder. It is recommended that family history includes also the search for leukemias or myelodysplastic syndromes, which occur with high frequency in many pedigrees with FDP-AML, *ANKRD26*-RT, and *ETV6*-RT.

Physical Examination

Physical examination aims to identify the abnormalities possibly associated with thrombocytopenia in syndromic ITs. As schematized in Fig. 1, these defects may affect all body districts, and examination should be therefore as complete as possible. The list of defects described in this figure is not exhaustive, and many other rarer abnormalities have been reported. Some defects are easily detected by general examination, while others are less evident, and their identification requires a targeted search. For instance, visual inspection is sufficient to detect the bilateral radial aplasia of TAR, while the radioulnar synostosis of RUSAT is suspected by the limited supination of the forearm. A few laboratory tests can complement the physical examination when searching for specific organ dysfunctions: urinalysis, to show proteinuria as the first sign of renal involvement in *MYH9*-RD, and dosage of liver enzymes, which are elevated in approximately 50 % of patients with this disease.

Blood Film Evaluation

Microscope evaluation of blood cells is a powerful tool for suspecting ITs since most of them are characterized by morphological anomalies of platelets, leukocytes, and/or erythrocytes (Table 3).

Platelets The most common abnormality of platelets is enlarged size with remarkable anisocytosis. Examples of giant platelets, large platelets, and platelets with normal size are given in Figs. 3 and 4. Table 4 reports a classification of ITs according to platelet diameters based on the analysis of 376 patients with 19 different disorders (Noris et al. 2014a). Depending on platelet diameters and the percentage of platelets larger than 3.9 μm (nearly half the diameter of normal erythrocytes), this classification distinguishes ITs with giant platelets, with large platelets, with normal or slightly increased platelet size, and with normal or slightly

Table 3 Morphological abnormalities of blood cells at examination of peripheral blood slides raising the suspicion that thrombocytopenia has a genetic cause

Blood cell abnormality	Inherited thrombocytopenia
Platelets	
Platelet anisocytosis	Most inherited thrombocytopenias
Giant platelets and >40 % of platelets larger than half a red blood cell	*MYH9*-RD, biallelic BSS, *PRKACG*-RT
Small platelets and/or <10 % of platelets larger than half a red blood cell	WAS, XLT, CAMT, *CYCS*-RT
Agranular ("pale") platelets (with large platelets)	GPS, *GFI1b*-RT
Hypo-granular platelets (with normal-sized platelets)	*ANKRD26*-RT
Some platelets with one single giant granule	TCPT/JBS, *FLI1*-RT
Vacuolated platelets	XLTT
Leukocytes	
"Döhle-like" inclusions in neutrophils	*MYH9*-RD
Red blood cells	
Anisopoikilocytosis or anisocytosis	*GATA1*-related disorders, *GFI1b*-RT
Stomatocytosis	STSL
Anisopoikilocytosis with dacryocytosis	GPS

For abbreviations of disorders, see Table 1

decreased platelet size. Platelet size in ITP is largely overlapping with that observed in ITs with large platelets and with normal or slightly increased platelet size, so that peripheral blood films evaluation has little utility in distinguishing these conditions. At variance, the percentage of large platelets has good sensitivity and specificity in differentiating ITs with giant platelets or with normal/reduced platelet size from ITP: the finding of more than 40 % or less than 10 % of platelets larger than half an erythrocyte strongly supports the diagnosis of the former conditions and makes the diagnosis of the latter unlikely. Cell counters are less effective than peripheral blood film evaluation in distinguishing ITs from ITP because, as already discussed, they often fail to recognize very large platelets and therefore underestimate MPV in ITs with giant platelets. However, it has been shown that a MPV value 50 % higher than the average value in healthy subjects has good diagnostic accuracy in recognizing ITs with very large platelets (Noris et al. 2009). Conversely, the finding of a normal or slightly increased MPV does not exclude the presence of giant platelets, and examination of blood films is required.

Abnormality of platelet granules is another finding suggesting the genetic origin of thrombocytopenia (Fig. 3d–h). Reduced or absent granules associated with platelet macrocytosis are typical of GPS and *GFI1b*-RT, while a reduced number of granules in normal-sized platelets may be observed in *ANKRD26*-RT (Noris et al. 2011). Identification of even a very small proportion of platelets with one single giant granule supports the diagnosis of TCPT/JCB (Penny et al. 1995).

Finally, a large number of vacuolated platelets support the diagnostic hypothesis of XLTT (Fig. 3i) (Balduini et al. 2004).

Leukocytes Blood film evaluation may be diagnostic in *MYH9*-RD, because the presence of faint, light-blue inclusion bodies (Döhle-like bodies) in polymorphonuclear leukocytes associated with very large platelets is pathognomonic of this disorder (Fig. 4) (Savoia et al. 2010). Döhle-like bodies consist of aggregates of wild-type and mutant MYH9 protein, MYH9 mRNA, and ribosomes. Conventional, panoptic staining reveals only the largest aggregates, which are identified in 42–84 % of patients. However, immunolabeling for MYH9 protein (Fig. 4e–g) discloses that these inclusions are present in all neutrophils of all patients (with the only exception of the very rare patients with mosaicisms) (Kunishima et al. 2005). The finding of aggregates at immunofluorescence assay for MYH9 is specific for *MYH9*-RD and is currently used as a screening test for this disorder (Savoia et al. 2010).

Erythrocytes The morphology of red blood cells deserves to be considered since abnormalities of their shape and size are observed in *GATA1*-related disorders, *GFI1b*-RT, sitosterolemia, and GPS.

Identification of the Specific Disorder of Each Patient

Identification of the specific form of IT affecting each patient is traditionally a two-step process: first, analysis of clinical data and a few simple tests to raise a diagnostic suspicion and then, second, targeted sequencing of the candidate gene(s) to confirm the diagnostic hypothesis. Figure 5 reports a diagnostic algorithm used in many centers (Balduini et al. 2003; Noris et al. 2004) that has been now updated to include the newly discovered diseases.

Syndromic Forms

Medical history and physical examination are used to recognize the defects possibly associated with thrombocytopenia in syndromic ITs and therefore to raise a diagnostic suspicion to be confirmed by genetic analysis. A few instrumental or laboratory tests may be required to identify and/or characterize certain defects. For instance, radiological examination is needed to characterize the bone defects of some ITs (Thompson et al. 2001). If *MYH9*-RD is suspected, audiometric and ophthalmological evaluation are required to confirm the presence of sensorineural deafness and cataracts

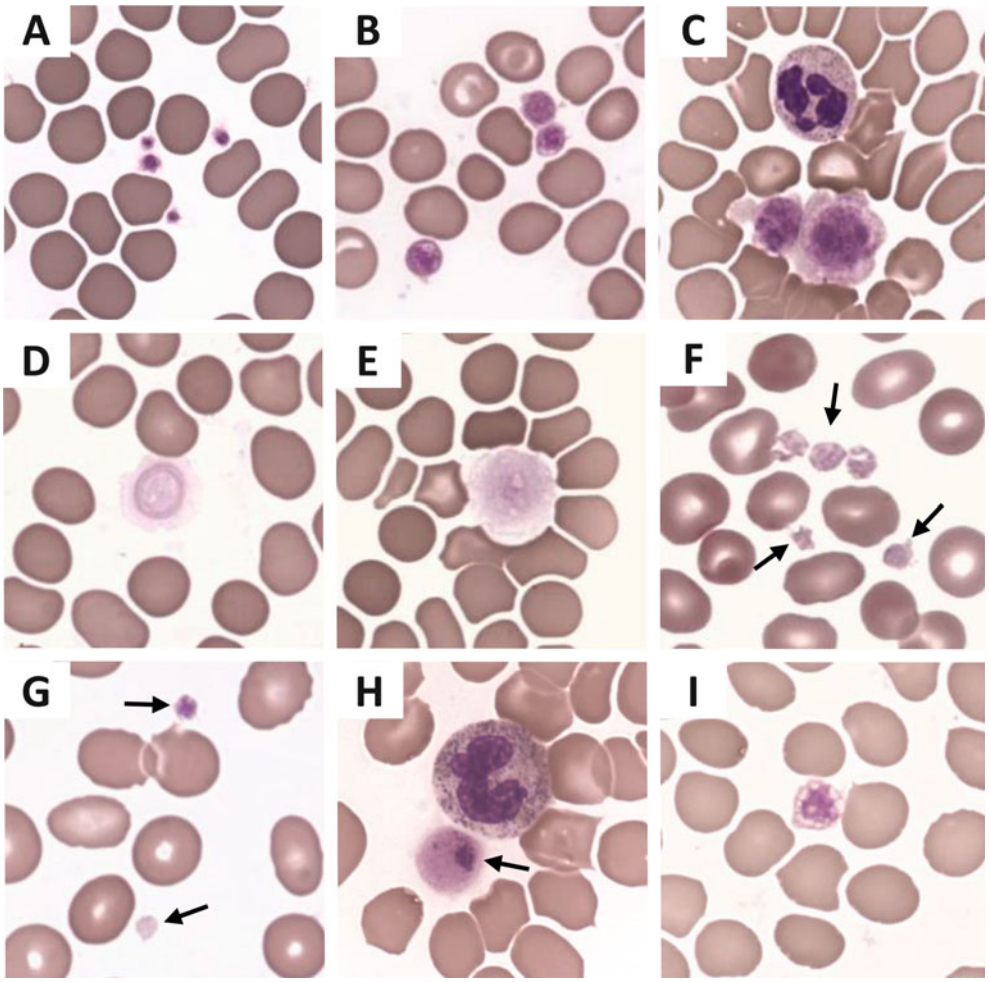

Fig. 3 Morphological abnormalities of platelets in inherited thrombocytopenias. Platelet size distinguishes forms with normal or nearly normal platelet size (**a**), with large platelets (**b**) and with giant platelets (**c**). The finding of large platelets that look "pale" in May–Grünwald–Giemsa-stained blood films is due to the absence of azurophilic granules (α-granules) and is highly suggestive of gray platelet syndrome (**d**, **e**), while a milder granule deficiency indicates *GF1Ib*-related thrombocytopenia (**f**). Granule deficiency in a variable proportion of platelets with normal or slightly increased size may be observed in *ANKRD26*-related thrombocytopenia (**g**, *arrows* indicate one platelet with normal α-granule content and one platelet without granules). The finding of platelets with a single giant granule is indicative of Paris–Trousseau thrombocytopenia/Jacobsen syndrome (**h**) even when only a very small percentage of platelets has this feature. Finally, large and vacuolated platelets have been described in *GATA1*-related diseases (**i**)

(Verver et al. 2016), respectively, while urinalysis is recommended to demonstrate proteinuria, which is the first sign of kidney damage (Pecci et al. 2008).

Non-syndromic Forms

After exclusion of syndromic forms, platelet size guides subsequent investigation (Pecci 2015).

Normal or Nearly Normal Platelet Size In case of normal, slightly increased, or slightly decreased platelet size, infants or children require bone marrow examination, because the absence/severe reduction of megakaryocytes indicates CAMT (Ballmaier and Germeshausen 2011). Regardless of age, bone marrow examination is due also in subjects with varying degrees of leukopenia and/or anemia, because the finding of bone marrow hypoplasia suggests *THPO*-RD. Once these two forms have been excluded, eight diagnoses are possible, and the transmission mode of thrombocytopenia in the family can help to narrow the diagnostic possibilities. However, the penetrance of mutations for thrombocytopenia may be incomplete in some dominant disorders, and normal platelet counts have been observed in few subjects with mutations that usually cause thrombocytopenia in *ANKRD26*-RT, FPD-AML, or *CYCS*-RT (Liew and Owen 2011; Noris et al. 2011).

Platelet Macrocytosis The diagnostic possibilities for subjects with large platelets are numerous. The diagnostic algorithm suggests to examine a peripheral blood film as the initial test. The finding of Döhle-like inclusion bodies in granulocytes of a patient with giant platelets is pathognomonic for *MYH9*-RD (Balduini et al. 2011a). Identification of platelet macrocytosis with most platelets looking "pale" due to a severe reduction in platelet azurophilic granules (α-granules) suggests GPS. A variable deficiency of granules in a proportion of platelets is compatible with *GFI1b*-RT (Stevenson et al. 2013, 2015b). The finding of red cell anisopoikilocytosis, often associated with anemia, indicates the ITs deriving from *GATA1* mutations (Millikan et al. 2011), while stomatocytosis associated with hemolytic anemia indicates sitosterolemia (Rees et al. 2005).

In case blood film evaluation does not identify abnormalities of blood cells other than platelet macrocytosis, three rather simple and inexpensive tests are used to further

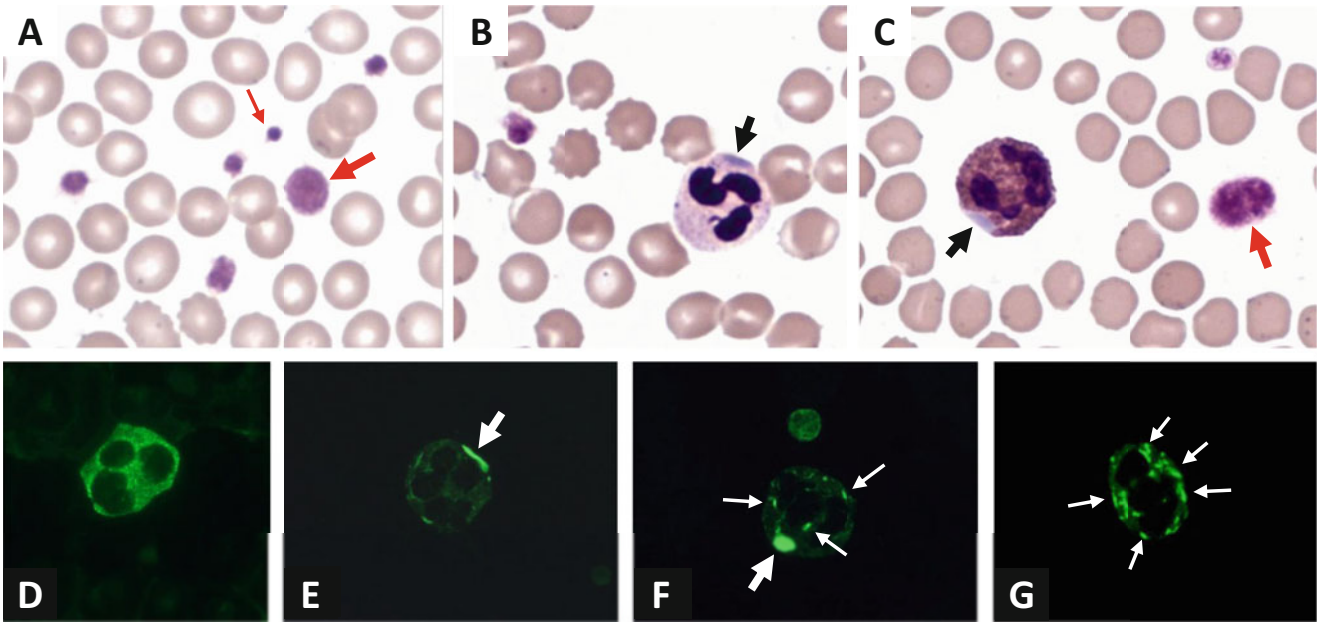

Fig. 4 Blood films in *MYH9*-related disease. (**a**–**c**) May–Grünwald–Giemsa-stained blood films; (**d**–**g**) immunofluorescence with antibodies against MYH9 protein. Platelet size in *MYH9*-related disease ranges from elements as large as red cells (*red*, *bold arrows* in **a** and **c**) to normal-sized elements (*thin arrow* in **a**). The faint, light blue, round-, or spindle-shaped inclusion bodies (Döhle-like bodies) located at the periphery of neutrophils and eosinophils shown (*black arrow*) in **b** and **c**, respectively, are typical of *MYH9*-related disease and are identified in about 50 % of patients. Immunofluorescence shows that these inclusions contain the *MYH9* protein clumped in a limited number of large or small spots (*bold* and *thin arrows*, respectively, in **e**–**g**) instead of being distributed homogeneously in the cytoplasm as in controls (**d**). Immunofluorescence analysis recognizes these inclusion bodies in almost all patients with *MYH9*-related disease and is therefore an excellent screening test for this condition

drive the diagnostic process. In vitro platelet aggregation after stimulation with ristocetin is markedly reduced or absent in patients with biallelic BSS: this diagnosis is confirmed by flow cytometric demonstration of severely reduced or undetectable GPIb/IX/V complex on the surface of platelets (see below) (Savoia et al. 2011; Berndt and Andrews 2011). Conversely, platelet aggregation in response to low ristocetin concentrations (0.3–0.4 mg/mL) that do not produce any effect in normal platelets suggests PT-VWD. In this condition, platelets often aggregate spontaneously in vitro upon stirring (Othman and Emsley 2014; Federici 2009). Of note, these abnormalities of in vitro platelet aggregation are observed also in type 2B von Willebrand disease (2B-VWD), which is clinically indistinguishable from PT-VWD. For differentiating these two conditions, we have to consider that in PT-VWD the abnormal platelet response to ristocetin derives from a defect of GPIbα that increases its affinity for VWF, while in 2B-VWD it is the consequence of mutations in VWF that increase the affinity of this molecule for GPIbα. Thus, plasma of subjects with 2B-VWD increases the response of normal platelets to ristocetin, while platelets of subjects with PT-VWD aggregate in response to low ristocetin concentration also when resuspended in normal plasma. Flow cytometry instead of platelet aggregation can be used to differentiate these two conditions by mixing tests (Giannini et al. 2010).

As discussed, immunomorphologic analysis for the MYH9 protein on blood slides is a powerful tool for diagnosing *MYH9*-RD, with sensitivity and specificity near to 100 % (Savoia et al. 2010).

Finally, flow cytometry for platelet surface glycoproteins is useful to confirm the diagnostic suspicion of biallelic BSS aroused from the study of platelet aggregation, as well as to raise the suspicion of monoallelic BSS or *ITGA2B*/*ITGB3*-RT. A reduction by about 50 % of the GPIb/IX/V complex in a subject with mild macrothrombocytopenia suggests a diagnosis of monoallelic BSS, while a reduced expression of the $\alpha_{IIb}\beta_3$ complex is compatible with *ITGA2B*/*ITGB3*-RT (Gresele et al. 2009).

New Diagnostic Approaches for Inherited Thrombocytopenias

The rapid advances in genetic techniques make currently feasible the targeted analysis of many different genes or even of the whole exome at once. Thus, it is now possible to search for mutations in all the genes whose mutations are

Table 4 Tentative classification of inherited thrombocytopenias based on platelet size. Most of inherited thrombocytopenias are classified according to the findings of Noris et al. (2014a)

ITs with giant platelets (mean platelet diameter >4 μm, more than 50 % of platelets larger than 4 μm)	*MYH9*-RD Biallelic BSS *PRKACG*-RT STSL
ITs with large platelets (mean platelet diameter >3.2 μm, more than 20 % of platelets larger than 4 μm)	*TUBB1*-RT GPS *FLNA*-RT *GFI1b*-RT Monoallelic BSS *ITGA2B/ITGB3*-RT *ACTN1*-RT *GATA1*-RD *SLFN14*-RT
ITs with normal/slightly increased platelet size (mean platelet diameter >2.6 μm, more than 5 % of platelets larger than 4 μm)	FDP-AML TCPT/JBS *FLI1*-RT *ANKRD26*-RT RUSAT *ETV6*-RT
ITs with normal/reduced platelet size (mean platelet diameter <2.6 μm, less than 5 % of platelets larger than 4 μm)	CAMT *CYCS*-RT WAS XLT TAR *THPO*-RT *FYB*-RT

For the forms discovered after publication of this study, data from literature have been used. For abbreviations of different disorders, see Table 1

known to result in ITs as a single-step diagnostic procedure instead of using a diagnostic algorithm to identify and prioritize one or a few genes to be sequenced. The single-step diagnostic approach might seem more convenient in terms of simplicity and, given the decreasing cost of massive parallel sequencing, even in terms of affordability. However, massive sequencing always finds a number of changes in a number of genes in each patient, and discriminating between nonpathogenic variants and variants that are causative of ITs may be a major problem. Thus, the best diagnostic approach to ITs is still a matter of investigation, and the skills available locally can influence the choice whether to use the multi- or the single-step methodology.

Another important question is whether the identification of the causative mutations is required in all cases. In fact, the number of known ITs and that of involved genes are continuously increasing, and, as discussed above, identifying the specific defect of each patient entails a considerable use of resources. In general, making a definite diagnosis is due whenever this translates into an advantage for the patient because this information assists physicians in defining prognosis and choosing the best treatment or follow-up regimen. On this basis, it is mandatory to make a definite, molecular diagnosis in patients with FDP/AML, *ANKRD26*-RT, and *ETV6*-RT because of their risk of developing hematological malignancies, in subjects with CAMT because of their constant evolution into bone marrow aplasia, in WAS/XLT because of the efficacy of specific treatments (see below), and in *MYH9*-RD because of the risk of developing kidney failure. Moreover, the identification of the causative mutation in *MYH9*-RD is important for defining the risk of non-congenital manifestations based on the well-established genotype–phenotype correlations (Pecci et al. 2014a; Verver et al. 2016). Finally, it has been shown that the THPO mimetic eltrombopag increases platelet count in this condition. Since FDP/AML, *ANKRD26*-RT, *ETV6*-RT, CAMT, and WAS are among the few ITs without platelet macrocytosis, it is recommended to diagnose at the molecular level subjects with an IT with normal or nearly normal platelet size. Moreover, it is recommended that patients with inherited macrothrombocytopenias receive the screening immunofluorescence test for *MYH9*-RD and, in case of positivity, undergo mutation screening in *MYH9*. The need for a molecular diagnosis is less stringent for the other forms of IT, although the identification of the causative mutations enables prenatal diagnosis and makes genetic consultation more effective.

Therapy

A comprehensive management of patients with ITs aims not only to prevent bleedings and stop hemorrhages once they occurred but also to take care of the other congenital and acquired defects possibly associated with thrombocytopenia.

General Measures for Preventing Bleeding

Patients must be informed that many drugs, as aspirin and other nonsteroidal anti-inflammatory drugs, some antidepressants, antibiotics, and anesthetics, impair platelet function and therefore increase the risk of bleeding. These medications should be administered only after a careful evaluation of the risks/benefits balance (George and Shattil 1991). Subjects with the most severe forms of IT should avoid activities at high risk of trauma, such as contact sports. Another important measure is to advise patients to perform accurate oral hygiene and to make regular dental visits to prevent future invasive dental procedures. Administration of oral contraceptives is usually effective in preventing or controlling menorrhagia.

It is important to emphasize that even patients with ITs are at risk of developing thrombotic events (Girolami et al. 2013). The decision to use antithrombotic prophylaxis must

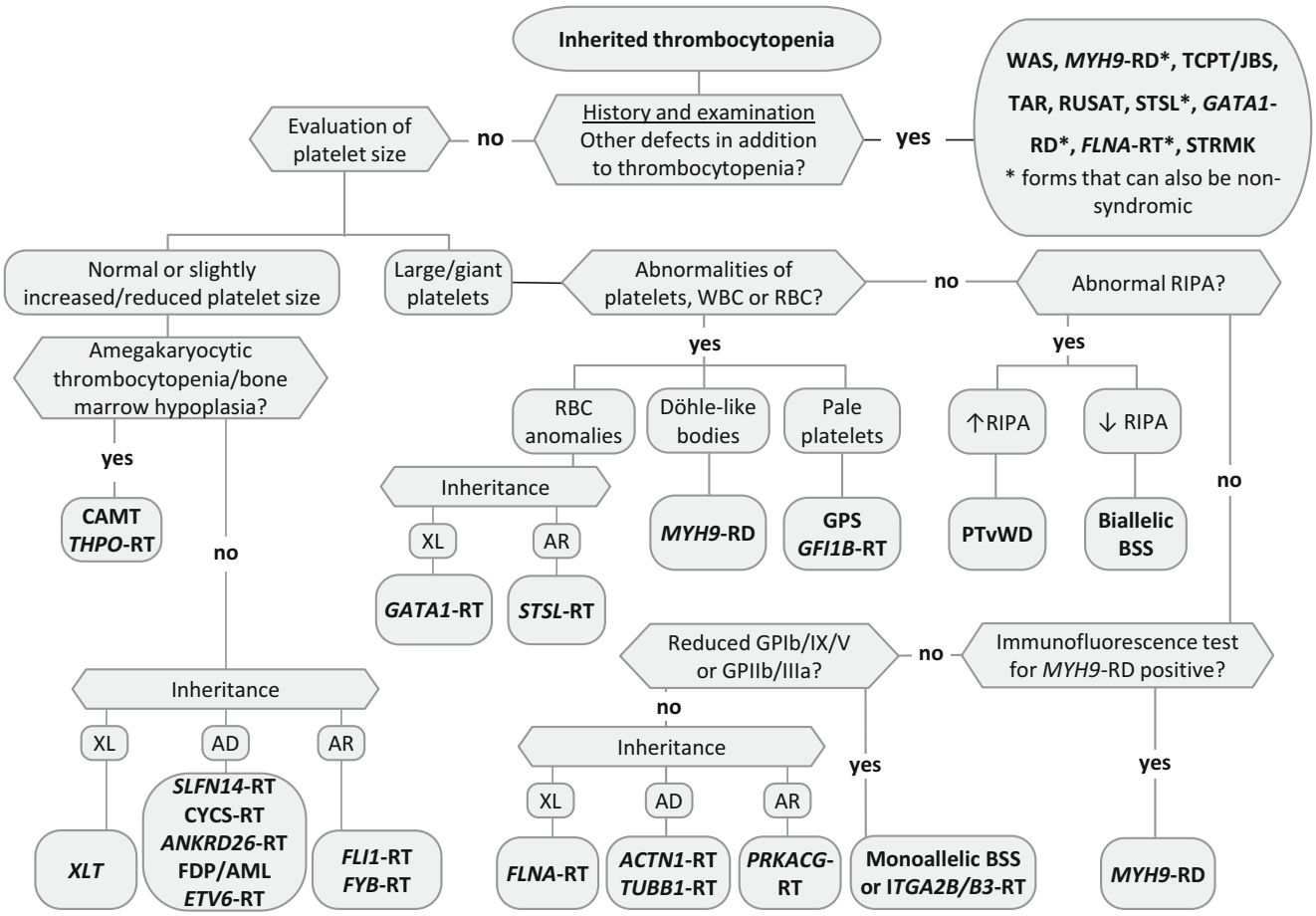

Fig. 5 Diagnostic algorithm for inherited thrombocytopenias. History and examination are used for diagnosis of patients with syndromic forms, whereas morphological evaluation of blood films and three laboratory tests guides the diagnostic process in non-syndromic forms. Definitive confirmation of the diagnostic hypotheses requires sequencing of candidate genes. For abbreviations of disorders, see Table 1; *RIPA* ristocetin induced platelet aggregation

be taken after a careful evaluation of the risk/benefit ratio based on the overall clinical picture of each patient.

Preparation to Hemostatic Challenges

Platelet count and bleeding history are the elements to be considered to decide how to prepare patients to hemostatic challenges. Subjects with more than 100×10^9 platelets/L do not need any precautionary treatment if their platelet function is not severely defective, while those with less than 50×10^9 platelets/L need prophylactic treatment. Bleeding history and typology of the hemostatic challenge direct the management of patients with $50–100 \times 10^9$ platelets/L (Tosetto et al. 2009). For instance, subjects with no or very mild previous bleeding events can undergo surgery or delivery without any support, while those with previous hemorrhages require prophylactic treatments. Prophylactic treatments should be considered also in all patients who need major surgeries, especially at critical sites as eye or nervous system.

Platelet Transfusions

Prophylactic platelet transfusions are effective in reducing the risk of bleeding, even if their administration does not ensure that the patient will not bleed. Platelets from HLA-matched donors should be used whenever possible to prevent or overcome alloimmunization and refractoriness to subsequent platelet infusions. Subjects with biallelic BSS who completely lack the platelet GPIb/IX/V complex can develop isoimmunization against the components of this complex. In this case, immunosuppression and/or plasmapheresis to clear antibodies can restore the efficacy of platelet transfusion (Liumbruno et al. 2009).

Antifibrinolytic Drugs

Successful systemic administration of antifibrinolytic agents, such as tranexamic or epsilon-aminocaproic acid, has been

reported for covering minor surgery in a few patients with ITs (Althaus and Greinacher 2009; Pecci et al. 2014b). However, no clinical study on the effectiveness of these drugs in ITs has been performed, and this treatment should be considered as empirical. Antifibrinolytics are contraindicated in hematuria given the risk of clots formation in the renal collecting system (Kumar et al. 2013).

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is an approved treatment for mild hemophilia A and type 1 von Willebrand disease due to its ability to stimulate the release of VWF from endothelial cells. DDAVP also enhances the procoagulant activity of platelets (Colucci et al. 2014), and this observation could provide a possible explanation for the successful use of the drug for covering minor surgery in some patients with mild ITs (Tosetto et al. 2009; Sehbai et al. 2005). However, clinical studies on the efficacy of DDAVP in these conditions are still lacking. Though rarely, DDAVP is associated to important side effects, such as hypotension, tachycardia, fluid retention, and hyponatremia, and for this reason it should be used with caution in patients with ischemic or congestive heart disease or heart failure, as well as in infants.

Activated Recombinant Factor VIIa

Activated recombinant Factor VIIa (rFVIIa) promotes thrombin generation by both tissue factor-dependent and tissue factor-independent mechanisms and is currently approved for treating patients with Glanzmann thrombasthenia (Poon 2007). In the setting of ITs, it has been successfully used for preparing to invasive procedures a few subjects with biallelic BSS (Tefre et al. 2009; Hacihanefioglu et al. 2007) and one girl with TAR (Coppola et al. 2007). Thromboembolic events are potentially severe side effects of rFVIIa.

Short-Term Eltrombopag

The thrombopoietin mimetic eltrombopag is licensed for therapy of chronic ITP and chronic hepatitis C, as well as treatment of severe aplastic anemia refractory to immunosuppressive drugs. A short-term course of eltrombopag has proved effective in transiently increasing platelet count in patients with *MYH9*-RD and less than 50×10^9 platelets/L (Pecci et al. 2010). Eltrombopag increased platelet concentration to values around or above 100×10^9/L in 8 of 12 patients, with minor increases in the remaining subjects. Importantly, eight of the ten patients with spontaneous bleeding at baseline achieved complete remission of hemorrhages. Based on these findings, short-term eltrombopag administration has been successfully used for preparing to surgery one adult and one pediatric patient with *MYH9*-RD (Pecci et al. 2012; Favier et al. 2013). It is presently unknown whether short-term eltrombopag is effective for preparing to surgery patients with ITs other than *MYH9*-RD.

Stopping Hemorrhages

Local measures are first-line treatment for mild or moderate mucocutaneous bleedings. Endoscopic electrocautery and nasal packing are recommended for stopping epistaxis. Compression and application of gelatin sponges or gauzes soaked in tranexamic acid can be effective in controlling bleeding from superficial wounds. Suturing can stop hemorrhages from accidental or surgical wounds (for instance, bleeding after tooth extraction). Mouthwash with tranexamic acid may be useful for gingival bleeding.

In case local measures are not possible or do not work, platelet transfusion (see above) is the measure to be used first. In case of refractoriness to platelet transfusions, rFVIIa has been proved effective in a small number of patients with BSS (Tefre et al. 2009; Hacihanefioglu et al. 2007). There is no experience with the use of this drug in other ITs. The role of antifibrinolytic drugs and DDAVP for stopping hemorrhages remains to be ascertained.

Treatments to Stably Increase Platelet Count

Allogeneic hematopoietic stem cell transplantation (HSCT) and gene therapy can potentially cure ITs, while splenectomy and long-term administration of TPO mimetics have the potential to stably increase platelet count in some disorders.

Allogeneic Hematopoietic Stem Cell Transplantation

The median life expectancy of patients with WAS is about 15 years, and all CAMT patients die for bone marrow aplasia during infancy. HSCT is the treatment of choice for both WAS and CAMT as it can cure all the aspects of these severe disorders.

Concerning WAS, 5-year overall survival of 122 patients who underwent HSCT after the year 2000 was 100 % for children transplanted from HLA-matched siblings and 90 % for those transplanted from HLA-matched unrelated or mismatched related donors (Moratto et al. 2011). Patients transplanted from unrelated donor after the age of 5 years had the worse outcome, with a 5-year survival around 75 %. Thus, even if early transplantation is desirable, treatment from unrelated donors should be offered also to older patients.

HSTC should be considered also for patients with severe, syndromic forms of XLT, in that a retrospective study of 24 patients revealed that 20 of them were alive without

serious posttransplantation events after a median follow-up of 50 months. However, four deaths for infections were reported (Oshima et al. 2015).

For what it concerns CAMT, near 60 children treated by HSCT have been reported in the literature. The overall long-term survival rate was 80 %, with 72 % of patients having normal donor hematopoiesis and good quality of life (Ballmaier and Germeshausen 2011). HSCT-related mortality was 8 % in children transplanted from related donors and 23 % in those transplanted from unrelated donors (Woods et al. 2014).

HSCT may be considered also in patients with severe forms of biallelic BSS with spontaneous, life-threatening hemorrhages, especially when they developed refractoriness to platelet transfusions. Two children and one adult received HSCT from HLA-identical siblings (Locatelli et al. 2003; Rieger et al. 2006), and all of them were cured.

Gene Therapy

Transplantation of autologous, gene-corrected hematopoietic stem cells is in principle an effective alternative to HSCT in patients with severe ITs. Gene therapy has been utilized so far only in patients with WAS. The first clinical trial used retroviral-transduced hematopoietic stem cell and obtained partial or complete resolution of immunodeficiency, autoimmunity, and bleeding diathesis. However, it was associated with a very high risk of insertional mutagenesis, with activation of several proto-oncogenes leading to leukemia in seven of the nine evaluable patients (Braun et al. 2014). Much better results have been obtained with lentiviral-based gene therapy. A total of 11 patients were treated at three European centers: ten of them obtained stable engraftment with improvement in platelet count and complete reconstitution of the immune function, while one patient died of infectious disease. Importantly, no evidence of vector-related toxicity was observed (Aiuti et al. 2013; Hacein-Bey Abina et al. 2015; Pala et al. 2015).

Splenectomy

Splenectomy does not induce any stable benefit in ITs, with the notable exception of WAS/XLT.

Analysis of the outcome of spleen removal in 41 XLT patients revealed that the cumulative incidence rate of serious bleedings was reduced, but there was a significantly higher incidence of severe infectious events (two fatal) (Albert et al. 2010). Overall survival in splenectomized patients was not significantly different from that of subjects not undergoing splenectomy. Thus, the risk–benefit balance of splenectomy should be weighed in each patient, taking care to administer anti-infective prophylaxis to subjects receiving this treatment.

In WAS, splenectomy should be avoided in patients candidate to HSCT, as it increases the risk of severe infection after transplantation (Ozsahin et al. 2008). The risk–benefit balance seems to favor splenectomy in WAS patients who cannot be transplanted. Thirty-nine patients with no suitable bone marrow donors underwent splenectomy, with most of them receiving prophylactic antibiotics to minimize the risk of sepsis (Mullen et al. 1993). Almost all patients achieved normal platelet counts, and the rate of serious bleeding was reduced nearly sevenfold. However, five patients died of sepsis. Median survival in the non-transplanted splenectomy group was 25 years, compared with less than 5 years in non-transplanted and non-splenectomized patients.

Long-Term Eltrombopag

Long-term eltrombopag administration for achieving stable increase of platelet count has been tested in a series of eight patients with WAS/XLT and severe thrombocytopenia (Gerrits et al. 2015). Five subjects responded (achievement of at least one on-treatment platelet count higher than 50×10^9/L). More importantly, all the five responders obtained improvement of baseline spontaneous bleeding. No major adverse events have been recorded. However, the safety profile of prolonged administration of eltrombopag in WAS/XLT requires further studies, and chronic administration of this drug cannot yet be recommended.

Treatments for the Other Defects or Illnesses that Add to Thrombocytopenia

Patients with FPD/AML, *ANKRD26*-RT, and *ETV6*-RT are at risk of developing acute leukemias and myelodysplastic syndromes. When this happens, HSCT is an important part of the therapeutic armamentarium. In case a related donor is available, it is important to exclude that he too is affected by the same IT, because transplanting hematopoietic cells of affected relatives exposes patients to the risk of developing again a new hematological malignancy (Buijs et al. 2001).

Concerning the extra-hematological defects of *MYH9*-RD, four patients with renal involvement had their proteinuria stably reduced by angiotensin receptor blockers and/or angiotensin-converting enzyme inhibitors (Pecci et al. 2008). The possible, favorable effect of this treatment was confirmed by more recent observations (Sekine et al. 2010; Oh et al. 2015). However, it is premature to conclude that these drugs prevent or delays deterioration of renal function. Dialysis or kidney transplantation is required in individuals with end-stage renal disease. Cochlear implantation is effective in restoring hearing capacity when deafness severely affects the quality of life: restoration of an excellent hearing function was obtained by eight of ten subjects receiving this treatment, while one patient had a slightly worse performance that could be explained by the very long duration of severe deafness. Only one patient did not significantly

improve (Pecci et al. 2014a, b). Patients with *MYH9*-RD should be monitored for the possible development of cataract, and the standard surgery should be carried out when indicated.

In patients with ITs associated with congenital limb deformities, reconstructive orthopedic surgery allows children to return to most activities of daily living (Al Kaissi et al. 2015).

Take Home Message

- Inherited thrombocytopenias are more frequent than previously thought. Since bleeding tendency is often mild or even absent, thrombocytopenia may be identified incidentally in childhood or adult life, and affected patients are at risk of misdiagnosis with ITP.
- Medical history (to identify other affected family members), physical examination (to recognize the additional defects of syndromic forms), and blood film examination (to detect the characteristic abnormalities of blood cell morphology) are the most important tools to suspect the genetic origin of thrombocytopenia.
- Making a definite diagnosis is important to recognize the forms of inherited thrombocytopenias that predispose to the acquisition of additional diseases, like hematological malignancies and renal failure.
- Specific treatments for some forms of syndromic and non-syndromic inherited thrombocytopenias have been identified.

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Immune Thrombocytopenia: Where Are We Now?

Nichola Cooper and James Bussel

Abstract

This review focuses on megakaryocyte development, regulation of platelet production, and the role of thrombopoietin (TPO) in relation to thrombocytopenia and correction of thrombocytopenia in immune thrombocytopenia (ITP). It appears that molecules (antibodies) and cells (T cells) that attack platelets are also directed against megakaryocytes and, thus, ITP is characterized by not only by accelerated platelet destruction but also by inhibition of platelet production. There is also a relative TPO deficiency, meaning that correction can often be achieved by administration of TPO receptor agonists. Current understanding of the pathology of ITP is extremely complicated. It seems that T helper cells (CD4) are required, even to make antibodies to platelets. However, initial studies of plasma infusion from ITP patients into normal subjects suggested that, in 10 of 26 cases, infusion of such plasma does not result in thrombocytopenia. It is possible that, in some of these outliers, all the antiplatelet antibody is on the platelets themselves and there is no free antibody in the plasma. However, clinical experience indicates that cytotoxic and other T cells are heavily involved and that there are almost no functions (receptors, cell to cell interactions, cytokine releases) of T cells that have not been demonstrated to be abnormal in ITP. Several studies with the beta T cell receptor, demonstrating important olioclonal and clonal abnormalities, have reinforced this.

Treatment of ITP remains very complicated and uncertain after a course of steroids (even there, the choice of dexamethasone versus prednisone has not been settled). Rituximab-based treatment and use of TPO receptor agonists have strong studies favoring their use, but other studies show limited likelihood of a good response in patients. It is easier to increase the platelet count in the short term than to achieve the "Holy Grail" of a cure.

Introduction

Definition of ITP

ITP initially stood for idiopathic thrombocytopenic purpura. After being called immune thrombocytopenic purpura as a result of the studies by Shulman et al. (1965), ITP now stands for immune thrombocytopenia. The purpura was dropped when it was recognized that some thrombocytopenic patients did not have manifestations of bleeding. Other abbreviations included AITP (autoimmune thrombocytopenic purpura).

The current definition (unlike the initial one) excludes non-immune thrombocytopenias such as the inherited thrombocytopenias. Although the concept of autoimmune

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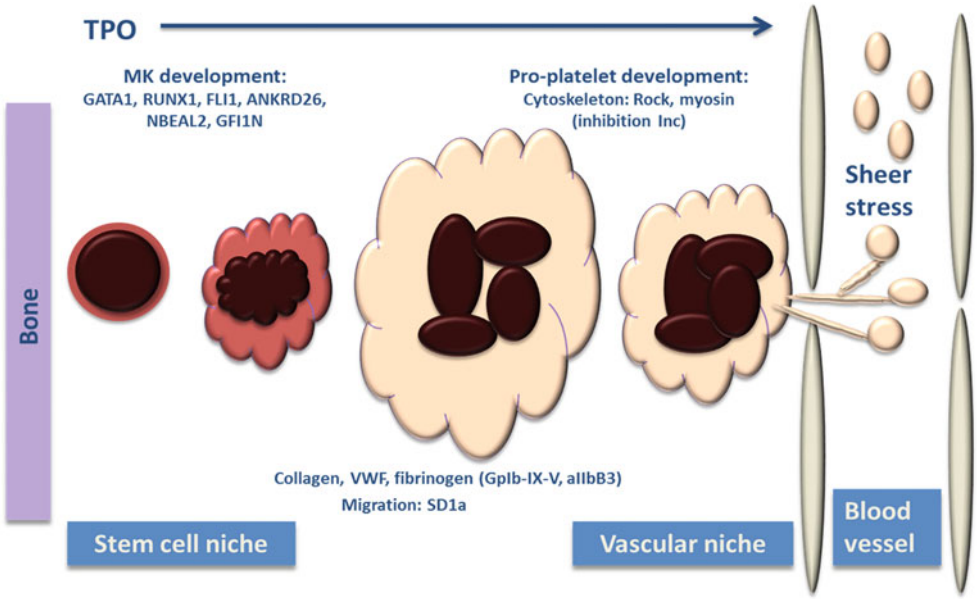

Fig. 1 Megakaryocyte (*MK*) maturation and platelet development in ITP. Differentiation of megakaryocytes and mechanism of platelet release, with incomplete listing of key mediators. This figure is adapted from Pecci A, Balduini CL (2014) Lessons in platelet production from inherited thrombocytopenias. Br J Haematol 165(2):179–92

(autoantibody-mediated) thrombocytopenia is very clear and unanimously agreed upon, ITP remains a diagnosis of exclusion because of the lack of utility of platelet antibody testing. This is complicated because, for example, the only way to know that an apparent case of ITP is primary and not, for example, secondary to hepatitis C, CMV, *H. Pylori*, CVID, or CLL is to run tests to exclude these possibilities. However, such extensive testing is far from the standard of care. Which epidemiologic, clinical, or laboratory features to use as the basis for performing certain additional tests has never been thoroughly explored or validated.

The only way to confirm ITP positively is by a good response to ITP-specific therapy. The classic example is a dramatic but transient overnight platelet increase in response to intravenous gamma globulin (IVIG), but even a rapid and substantial response to steroids is very suggestive of ITP. These responses, if present, are believed to be useful if there is a response but do not distinguish primary from secondary ITP.

At present, there is clear consensus that there is little benefit of anti-platelet antibody testing to include or exclude the diagnosis of ITP. In the future, if preliminary findings are confirmed and the consensus shifts, then distinction of types of ITP between those with predominant anti-platelet antibodies directed against GPIb-IX-V and those with antibodies primarily directed against $\alpha_{IIb}\beta_3$ may become clinically important; or those with antibodies compared with those lacking antibodies.

Platelet Production and Regulation of Thrombopoietin Levels

Megakaryocyte development is driven by many different transcription factors (as shown in Fig. 1), a process further complicated by migration of megakaryocyte progenitors, which start in the stem cell niche and must get to the vascular niche. Migration is dependent on SDF1α and relies on collagen, von Willebrand factor, and fibrinogen interacting with the platelet and megakaryocyte counter-receptors GPIb-IX-V and $\alpha_{IIb}\beta_3$. Once the pro-platelets are extended into the blood vessel, shear forces from blood flow cause them to fragment off (see Italiano (2017) for more details). Even if they are still platelet aggregates, they become single platelets after they arrive at the pulmonary capillaries.

Traditionally, antibodies to platelets have been thought to be directed to $\alpha_{IIb}\beta_3$ and GPIb-IX-V. However, an interesting feature is that the Ashwell–Morell receptor in hepatocytes might play a role in platelet regulation. Antibodies to GPIb-IX-V can cause externalization of neuraminidase, which then desialylates GPIb on platelets. These desialylated platelets, like aged platelets, are cleared in the liver by interaction with the Ashwell–Morell receptor. Data from Karen Hoffmeister's group suggests that, via a receptor that is similar to that of interleukin(IL)-6, these platelets then stimulate production of thrombopoietin (TPO) via the JAK2–STAT3 signaling cascade (Grozovsky et al. 2015). However, whether this happens rarely, all the time, or somewhere in between remains to be clarified (Fig. 2).

There is no correlation between platelet counts and TPO levels. Instead, the TPO levels seem to depend on the numbers of megakaryocytes in the bone marrow (Fig. 3). This is similar to the regulation of granulocyte colony-stimulating factor but not erythropoietin levels (Corbacioglu et al. 2000). Thrombocytopenic patients with aplastic anemia have very high TPO levels, whereas patients with ITP and very low platelet counts have only slightly elevated or normal circulating TPO (Kuter et al. 1994). This is the so-called Kuter hypothesis. What is less clear are the mechanisms of regulation, up or down, of TPO

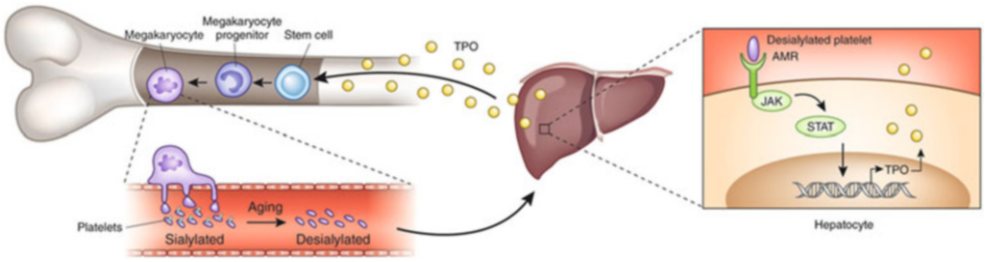

Fig. 2 Old and desialated platelets are recognized by the Ashwell–Morell receptors (*AMR*) in hepatocytes, causing thrombopoietin production. Physiology of the Ashwell–Morell receptor and its role in TPO production via the JAK-STAT pathway. Taken from Kile BT (2015) Nat Med 21:11–12

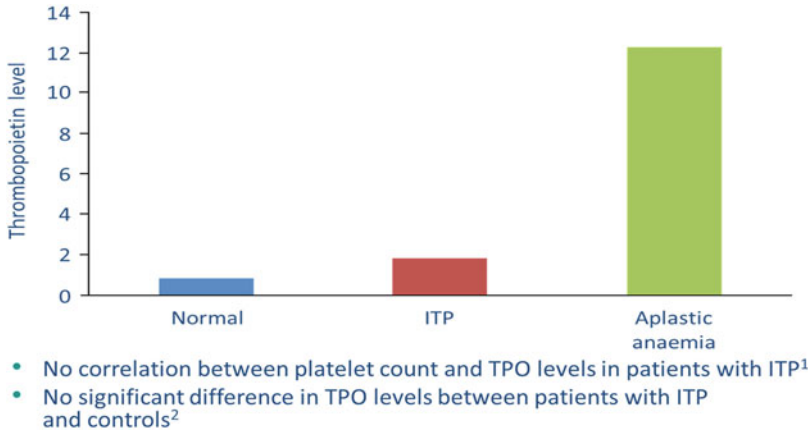

Fig. 3 Kuter hypothesis of TPO regulation. The role of platelet count and marrow megakaryocyte number in determining thrombopoietin level: low platelet count in ITP (in which there are ample numbers of megakaryocytes in the marrow) is consistent with TPO binding to TPO receptors and thus a reduction in the level of TPO to normal or near normal. Adapted from Kosugi S et al (2003) Br J Haematol 93:704–706; Aledort LM et al (2004) Am J Hematol 76:205–213

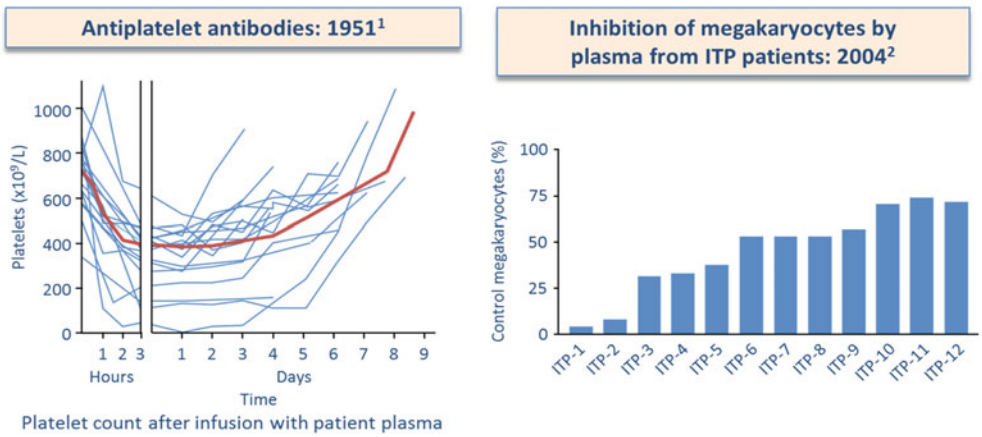

Fig. 4 ITP is an autoantibody-mediated disease. (**a**) Original experiment by Harrington et al. (1951), demonstrating the thrombocytopenic effect of plasma from ITP patients when infused into normal recipients. (**b**) Antibodies from patients with ITP inhibit the growth of megakaryocytes in culture (McMillan et al. 2004)

production. Current studies suggest that the role of inflammation is mediated primarily by IL-6 in the liver.

Autoimmunity in ITP

How do we know that ITP is an autoimmune disease? The classic studies of Harrington (Fig. 4a) showed that infusion of ITP patient's plasma into normal controls mostly (but not always) causes thrombocytopenia in the recipients (Harrington et al. 1951). Follow-up studies have shown that this effect appears to be antibody mediated. Bob McMillan's group and Mei Chang in Diane Nugent's group (Fig. 4b) showed that plasma from ITP patients inhibited growth and development of megakaryocytes to variable extents; the plasma from different patients had different degrees of effect in both studies (Chang et al. 2003; McMillan et al. 2004). It thus appears clear that antiplatelet antibodies react not only with platelets but also with megakaryocytes. Whether this is important to the pathogenesis of ITP in all, most, or some cases is not clear.

What do we know about T cells in ITP? There have been a multitude of studies demonstrating a multitude of abnormalities of T cells (Semple et al. 1991, 1996). One

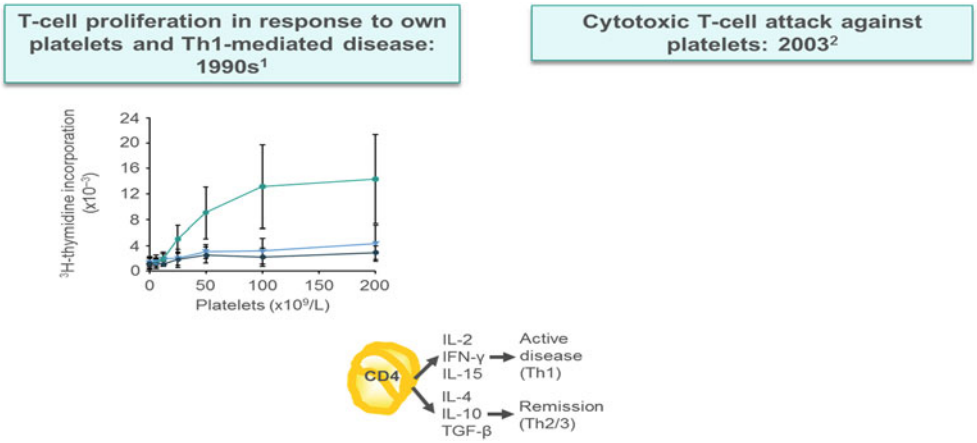

Fig. 5 Role of T-cell-mediated platelet destruction in the immune pathology in ITP. (**a**) T cell proliferation in response to a patient's own platelets (Semple et al. 1991). (**b**) Cytotoxic T cells play a role in destruction of platelets in active ITP (Olsson et al. 2003) (CD4 skewing to Th1 should be figure C, and comes from Semple JW and Provan D (2012) Curr Opin Hematol 19:357–362)

set involves the abnormalities of T cells as complex immune regulators of the immune response. The most sophisticated involve analyses of newly discovered cytokines or of cell populations such as follicular B cells in the spleen and their role in anti-platelet antibody regulation and/or production. Other studies have similar levels of sophistication and are very complicated.

Perhaps the clearest and most uniform findings have involved T regulatory cells (Tregs). Evidence for their deficiency in both number and function has been confirmed in a number of studies (Bao et al. 2010). Similarly, their restoration in response to therapy has been invoked not only with TPO agents but also using several different therapies (e.g., rituximab) (Stasi et al. 2007). Tregs can suppress the interaction of dendritic cells with T helper cells and prevent T helper cells from interacting with B cells to make antiplatelet antibodies by reducing their proliferation.

A different case is investigation of cytotoxic T cells to determine their role in direct destruction of platelets and megakaryocytes. A classic study of cytotoxic T cells was carried out by Wadenvik's group. In a small number of patients with ITP, an in vitro natural killer-type assay showed that there was cytotoxicity to platelets, with lysis. Furthermore, the study pinpointed the mechanism (e.g., granzyme levels were increased) and showed that not only was there more lysis in patients with ITP than in controls but also more cytotoxicity with active ITP than for ITP in remission (Fig. 4b) (Olsson et al. 2003). Additional studies of this topic have been very limited.

In the 1990s, there were studies showing T cell proliferation in response to patients' own platelets and studies consistent with an active TH1-mediated disease driven by IL-2, interferon γ, and perhaps even IL-15 (Fig. 4a). To date, hundreds of studies have been performed demonstrating a confusing variety of abnormalities of T cells and their cytokines (Fig. 5).

The role of CD8 cells seems more complicated than just to attack platelets and megakaryocytes in patients with ITP. It appears that CD8 cells are necessary for megakaryocyte proliferation and, when CD8-positive T cells are co-cultured with megakaryocyte progenitors, more megakaryocytes develop, there is less apotosis, and fewer platelets, which is somewhat surprising (Li et al. 2007; Fig. 6). Similarly, it appears that dexamethasone can increase the number of CD8-positive T cells and increase Tregs, which is a surprising finding given the expected immunosuppressive effect of steroids.

Some of the complexities of the pathogenesis of ITP, in addition to those mentioned, include epitope spreading (presentation of new epitopes of the platelet glycoproteins so that there is a broader spectrum of anti-platelet antibodies) in macrophages, which may contribute to chronicity and failure to respond to ITP therapies; the role of anti-platelet antibodies; and the role of these antibodies in impairing megakaryocyte production and maturation and in reducing platelet production. In addition, there are many co-stimulatory molecules involved between macrophages as antigen-presenting cells, helper T cells, and B cells including CD40, CD40 ligand (CD154), CD80, and CD28. These and other interactions could help determine whether the disorder is serious or not so serious and whether the patient is likely to get better over time (Cines and Blanchette 2002).

Management of ITP

There are many challenges in managing ITP. Diagnosis can be complicated, although not most of the time. Classic diagnostic practice involves excluding all other causes and having a normal complete blood count, except for thrombocytopenia, and no findings such as hepatosplenomegaly or lymphadenopathy on physical examination. Response to

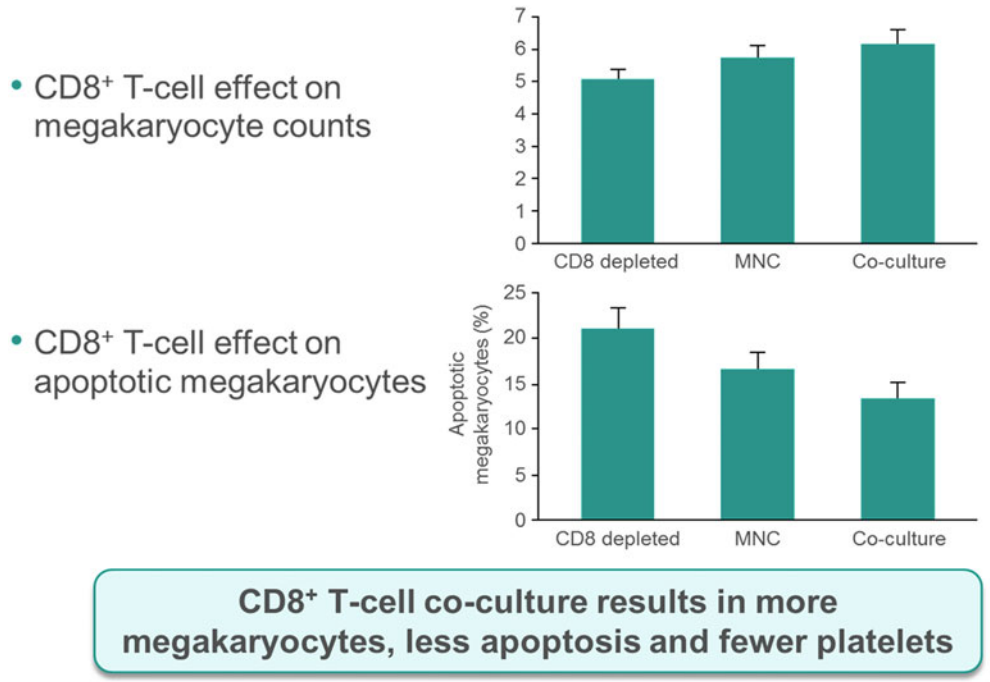

Fig. 6 The role of $CD8^+$ T cells in the bone marrow. Figure taken from Li S et al (2007) Br J Haematol 139:605–611

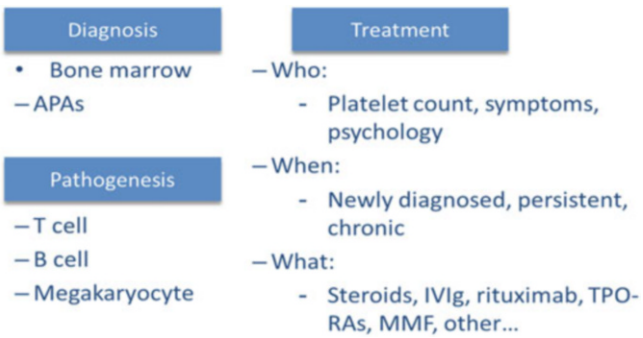

Fig. 7 Management of ITP. Diagnosis, pathogenesis, and treatment of ITP

treatment can also affect the diagnosis; for example, if a patient receives IVIG (or steroids) and the platelets increase dramatically, albeit transiently, then that strongly suggests that this is a case of ITP. It should be noted, however, that such a response (as mentioned in Sect. "Introduction: Definition of ITP") does not differentiate secondary from primary ITP (Fig. 7).

Pathogenesis can be very complicated and treatment decisions involve many different factors. These factors include the platelet count, the degree of symptoms especially bleeding and fatigue, and how the patient feels about treatment in general and a given treatment in particular. The risk of bleeding is an important factor for some patients (e.g., construction workers or athletes), as is travel (e.g., consideration of what to do when a patient does not have their usual source of emergency back-up available). Patients may be treated differently depending on the duration of their disease (newly diagnosed, persistent, or chronic) and can be treated with a wide variety of agents, as listed in the 2010 consensus document (Provan et al. 2010).

Decisions about treatment are not clearly defined. For adults, most are either asymptomatic or have minor bruising and perhaps a few scattered petechiae, despite relatively low platelet counts. Treatment is mostly given for platelet counts below 30,000/μL. There is increased mortality in elderly patients with persistently low counts (Cohen et al. 2000). Certain studies in the past have demonstrated that morbidity and mortality can derive from complications of immunosuppressive treatment given at too high a dose for too long, especially too much prednisone for months or years (Portielje et al. 2001).

The primary goal of treatment is to avoid intracranial hemorrhage, although improving health-related quality of life (HRQoL) is now recognized as very important and is more relevant for many patients. Data from Cortellazzo et al. (1991), summarized by Cohen et al. (2000), show that the rate of fatal hemorrhage per year for patients aged 40–60 years is 0.012 per year (i.e., just over 1 in 1000); for those aged over 60 years, the mortality is 0.13 per patient year (13 per 1000), which is thought to be driven to a large extent by comorbidities and other medications (Cortelazzo et al. 1991). In addition, patients with ITP have an increased risk of venous thromboembolism. In a long-term study of romiplostim, it was demonstrated that strokes

Clinical situation	Therapy option
First line (initial treatment for newly diagnosed ITP)	Anti-D Corticosteroids: Dexamethasone, methylprednisolone, prednis(ol)one IVIg
Second line	Azathioprine Cyclosporin A Cyclophosphamide Danazol Dapsone MMF Rituximab Splenectomy TPO-RAs Vinca alkaloids
Treatment for patients failing first- and second-line therapies	**Category A: Treatment options with sufficient data** TPO-RAs **Category B: Treatment options with minimal data and considered to have potential for considerable toxicity** Campath-1H Combination of first- and second-line therapies Combination chemotherapy HSCT

Fig. 8 First and second line treatments in the consensus document. Treatments listed alphabetically (also by category for third line) divided by first, second, and third line from the 2010 consensus document (Provan et al. 2010)

occurred particularly in the over 70 year olds (Kuter et al. 2013).

For children, and possibly younger adults, treatment is required if the count is over 30,000/μL only in cases where there is bleeding, the patient requires surgery, the patient is on anticoagulation or antiplatelet agents, and possibly if other factors such fatigue and access to care are involved. In the 10,000–30,000/μL range, the issue is whether treatment is needed and, if so, what to treat. Factors to take into consideration are age, comorbidities, and past treatment, including how well it worked or did not work. For guidelines or information as of early June 2016, there is the international consensus report that was published in 2010 (Provan et al. 2010) and the second version of the American Society of Hematology (ASH) evidenced-based guidelines for ITP (Neunert et al. 2011). Both the consensus report and the ASH guidelines are under revision and new versions should appear within the next year (Fig. 8).

The consensus report lists front line treatments, which everybody agrees with although there are differences about which treatments to use and when. The most recent study showed that the platelet count went up quicker with dexamethasone than with prednisone, but long-term outcome (the current Holy Grail of ITP treatment) was not significantly different between dexamethasone and prednisone treatments (Wei et al. 2016). The choice of second line treatments is more complicated because there is not much evidence other than some randomized trials of thrombopoeitic agents compared with placebo and a very large amount of open-label, single-arm data for splenectomy. A "How I Treat" article in 2012 focused on the selection of second line agents (Ghanima et al. 2012). For other agents, the issues are their efficacy and the side effects. For third line treatment, the only option that has sufficient data and category A evidence is use of thrombopoietic agents (Bussel et al. 2006, 2007; Saleh et al. 2013; Kuter et al. 2013).

For second line treatments, common morbidity for persistent ITP is caused by infection or other steroid-related complications and the most prominent steroid-sparing agents are rituximab; immunosuppression with danazol, azathioprine, or mycophenolate mofetil; TPO receptor agonists (TPO-RAs; romiplostim or eltrombopag); and splenectomy.

Looking at rituximab, the only study that assessed long-term outcome looked at responders 1 year after the initial rituximab infusion (Patel et al. 2012) and showed that the long-term outcome (more than 3 years) was only a 21 % persistent complete response. All other responding patients (approximately another 20–30 %), whether the response was partial or complete, relapse and require additional treatment (Patel et al. 2012). This number (21 %) could represent the percentage of people who would have improved over time anyway.

As this result became more evident, efforts were made to supplement rituximab treatment, the choice being high-dose dexamethasone. The initial concept was that rituximab would deplete intravascular B cells and plasma cells would die off. However, plasma cells lived longer than had hitherto been believed to be the case and, therefore, rituximab alone did not result in full depletion of antibody-making cells . This was consistent with the lack of development of hypogammaglobulinemia with rituximab alone (Cooper et al. 2004). Borrowing from experience with myeloma treatment, it was thought that dexamethasone would affect the plasma cells and rituximab would affect the B cells and, thus, there would be a better response to combined therapy. Two initial studies explored the addition of one 4-day cycle of dexamethasone to the standard dose of rituximab, but both compared this combination to one cycle of dexamethasone alone rather than to rituximab alone (Zaja et al. 2010; Gudbrandsdottir et al. 2013). The results were good, but only newly diagnosed patients (the easiest to treat patients) were entered in the studies and long-term follow up is not

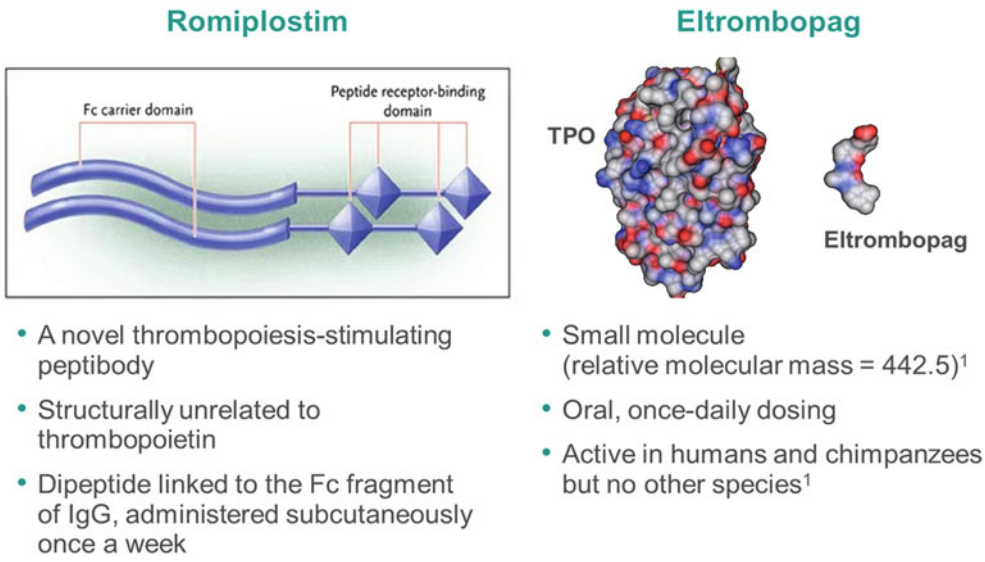

Fig. 9 Licensed TPO receptor agonists. The properties and physical attributes of the two licensed TPO agents, romiplostim and eltrombopag

available. More recent results using three cycles of dexamethasone and standard treatment of four doses of rituximab demonstrate that the only subset of patients with a very good cure rate(>70 %) are women with duration of their ITP of less than 1 year. This finding in women with ITP was supported by an identical result in adolescent girls (Chapin et al. 2016). It is, however, important to realize that, even in the groups that eventually relapse, as many as 50 % are in continuing response more than 2 years after the initial rituximab treatment. This combination provides good results at a high short-term (1–3 year) rate but the eventual relapse lessens the positive impact of this treatment scheme.

The next consideration was to use mycophenolate mofetil (MMF) as a second line treatment option. There are at least 11 ITP studies of MMF treatment, none of which are very large in size or randomized (Taylor et al. 2015). Overall, the response rates are about 50 % and MMF is well tolerated. Whether MMF is better than azathioprine is not clear. A newly issued "black box warning" (package insert for the USA) is that MMF can alter the levels of oral contraceptive hormones and therefore result in their failing to prevent pregnancy. MMF is best used in either mild to moderate disease or as a sequel to dexamethasone–rituximab to enhance cure rates (Chapin et al. 2016).

Splenectomy has become a very infrequently used treatment (Boyle et al. 2013). Over the period 1991–2009, in a US population, the rate of splenectomy fell from 30 to <10 % and is probably even lower today. Note that splenectomy offers the greatest chance for clinical remission and is potentially the most cost-effective treatment. Taking all comers with splenectomy, approximately 60 % of patients have durable remissions if the operation is not performed too late in the course of the disease (Kojouri et al. 2004). Currently, many patients refuse splenectomy if it is only going to work 60 % of the time. There are perioperative complications including infection, bleeding, and thrombosis (post-op anticoagulation treatment may be appropriate) and the long-term complications of infection with overwhelming post-splenectomy sepsis and thrombotic complications (Thomsen et al. 2010). A recent study presented at the European Hematology Association (EHA) congress in June 2016 by the group of Waleed Ghanima suggested that there was no increase in myocardial infarctions or pulmonary hypertension in patients with ITP who underwent splenectomy. However, there was a significant approximately 50 % increase in venous thrombosis and this translated into a significant increase in the incidence of stroke (Rørholt et al. 2016).

Turning to the thrombopoietic agents, we know that TPO drives the pathway from stem cells all the way to megakaryocyte proliferation (Kaushansky 1998). Its role in platelet release from megakaryocytes is uncertain. As discussed above, we do not know what fraction of TPO production is constitutive and how much it can be upregulated (e.g., by binding of desialated platelets to the Ashwell–Morell receptor) (Fig. 9).

Both romiplostim and eltrombopag were developed by screening of compounds. Romiplostim is composed of four small dipeptides attached to each other by disulfide bridges and to an Fc portion of an IgG_1 molecule to increase the half-life by allowing recirculation via FcRn. It is given once a week subcutaneously. In the USA, although not in Europe, it is still not approved for self-administration by the patient at home. Eltrombopag is given daily by mouth. Although there have been recent changes to the US package insert, we believe it should be taken on an empty stomach at least 2 h after eating and without eating for another 2 h afterwards. Furthermore, there should be no divalent cation intake (i.e.,

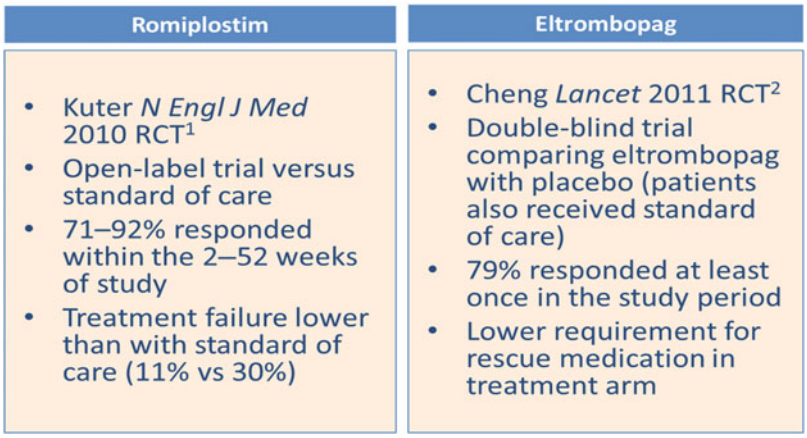

Fig. 10 Responses with TPO receptor agonists in adults (platelets $>50 \times 10^9$/L): Results from two randomized controlled trials (Kuter et al. 2010; Cheng et al. 2011). Description of two of the five large randomized controlled trials with these agents in adults with ITP (there are three large randomized trials in children with ITP)

calcium in milk or other dairy or other supplements for 4 h before and 4 h after). Most people therefore take it at bedtime, do not eat after dinner, and do not have dairy with dinner, but this is not always possible. Both romiplostim and eltrombopag are expected to cross the placenta and thereby affect fetal bone marrow, so their use in pregnancy is strongly discouraged. Both agents have been used at the very end of pregnancy to increase the platelet count in either refractory ITP patients or in those with inherited thrombocytopenias so that the platelet count is good enough for delivery and, hopefully, for epidural anesthesia (personal experience).

Both molecules share similar mechanisms of action although it is thought that they bind at different places, with romiplostim binding at the TPO binding site and eltrombopag potentially binding in the intramembranous portion. The difference in binding could underlie anecdotal findings in patients who respond to one agent but not the other (Khellaf et al. 2013). A theoretical advantage of the lack of eltrombopag binding at the TPO receptor site is that TPO can bind there, creating an additive or even synergistic effect, but this remains theoretical. Eltrombopag is a chelator and is inactive when it has chelated a cation, which is why calcium, magnesium, and iron inactivate it if ingested in proximity to the drug.

Figure 10 compares two randomized controlled trials (Kuter et al. 2010; Cheng et al. 2011). There are also a number of other randomized controlled trials with each agent. Adult ITP patients appear to respond 60–90 % of the time with either treatment and often can either discontinue or reduce the use of rescue treatments (i.e., platelet transfusion, steroids, IVIG, etc.) and/or discontinue or reduce the dose of concomitant medications (e.g., prednisone) after starting the thrombopoietic agent. Patients usually reduce bleeding in proportion to their platelet count increase and, overall, at least some of them have an improvement in their level of energy and/or HRQoL. Some of the latter may experience less bleeding and therefore less fear of bleeding but, in addition, thrombocytopenia has reversible organic effects on energy levels and vitality that have been amply demonstrated in a number of studies (McMillan et al. 2008).

Long-term results from both treatments have been collected and published or presented. The overall thromboembolic event rate was approximately 6 % for both agents over 6–8 years of treatment (Kuter et al. 2013; Saleh et al. 2013). There is still debate over the exact cause of the thromboembolisms. They appear to be a combination of the underlying disease, predisposition of certain individuals who have been "protected" by their thrombocytopenia, and the availability of an increased number of younger platelets. Bone marrow reticulin fibrosis, which raised considerable concern early on, seems to occur infrequently and does not cause any major issues (Ghanima et al. 2014). In addition, in patients who have discontinued treatment, the reticulin fibrosis has regressed in almost all cases (Ghanima et al. 2014). With eltrombopag, it seems that approximately 3 % of patients are unable to take it because of increases in transaminases and/or bilirubin, but most people with increased transaminases who discontinue eltrombopag can successfully resume it (Saleh et al. 2013). If eltrombopag can indeed cause cataracts, it appears to be a very small effect. Determination of a specific rate has been confounded by the many people with ITP who take steroids for prolonged periods of time, who are elderly, who may have smoked cigarettes, and who potentially have other risk factors for cataracts. There

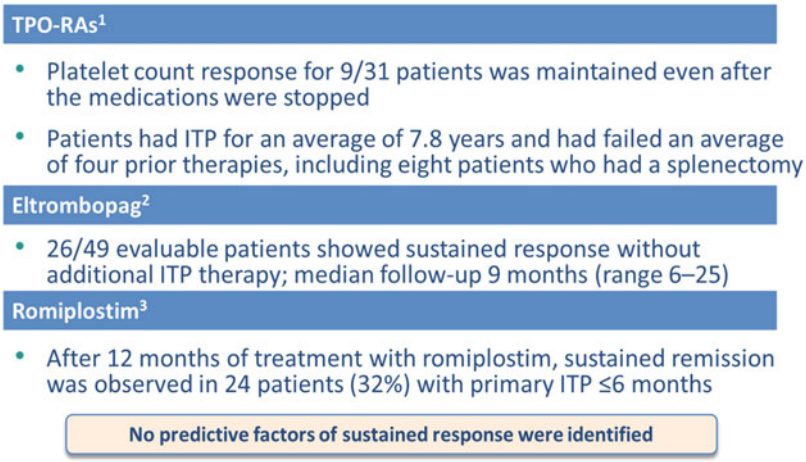

Fig. 11 Sustained remissions of ITP associated with the use of TPO receptor agonists

does not appear to be in ITP per se any induction of malignancy. If, however, a patient has myelodysplastic syndrome instead of ITP, there could be induction of blasts, which usually regress with discontinuation of medication (Bryan et al. 2010).

For both therapies, there are patients who, after a variable duration of treatment, can successfully discontinue a TPO agent and yet maintain an adequate count. What fraction of patients this is, how long they have had ITP, and how long they were on medication remains unknown. There is a weak tendency for patients who have been able to discontinue medication not to have been on TPO agents for a long time and not to have had ITP for a long time. Furthermore, even in adults, there can be spontaneous improvement over a number of years. This continues to be studied without clear resolution at this time, although one could hazard an estimate of 20–40 % improvement within 3 years of starting thrombopoietic agents (Fig. 11).

There is also the question of why remission should occur. Possibly, it occurs as a result of spontaneous improvement over time and the TPO agent is merely supporting the platelet count until "spontaneous remission or improvement" does occur. Alternatively, thrombopoietic agents may not be as immunologically inert as originally thought. This is consistent with platelets being part of the innate immune response (see Slaba and Kubes 2017). At least one study has showed the induction of Tregs, not just by number but also by function (Bao et al. 2010).

Figure 12 outlines one possible schema for an approach to ITP. Clearly, the great majority of the decisions in this setting are not informed by randomized controlled trials. However, an unequivocal issue here is not to overly prolong the use of steroids. If there is no early remission, the side effects of prolonged use of steroids outweigh their benefit. IVIG as a rescue medicine (i.e., to bring the count up quickly) is a reasonable early approach but has relatively little curative effect, although there may be a small amount with repeated use as demonstrated in children with ITP. In persistent ITP, MMF has been widely used. Other treatments are TPO agents or rituximab. The latter is more likely to work with dexamethasone and if used closer to diagnosis rather than further from it. Whether the benefit of adding three cycles of dexamethasone to rituximab outweighs the side effects is not clear. However, at this time, for women within 1 year of diagnosis, especially those who are early persistent, it seems very useful (Chapin et al. 2016). Other agents for chronic patients, in addition to splenectomy, include danazol, dapsone, azathioprine, and possibly even hydroxychloroquine if there are lupus-like features (Provan et al. 2010).

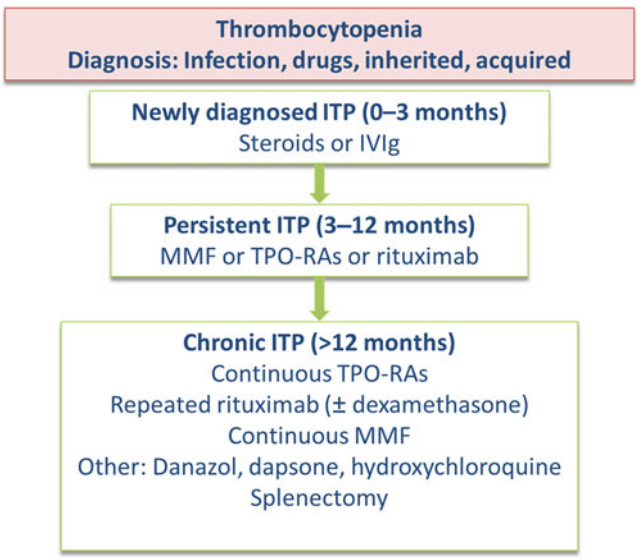

Fig. 12 A possible approach to ITP diagnosis and treatment

Pediatric ITP

Children with ITP are thought to have the "same" disease as adults. This may be most true for children with very persistent and chronic disease in which no spontaneous improvement occurs and standard treatments are sometimes ineffective. However, for most children with ITP, the ITP resolves spontaneously within 3–6 months. It is thought that many of these cases reflect "virally induced" ITP with antibodies to an infective agent that cross-reacts with platelets. As the infection clears and antibody titers fall, the platelet count returns to normal. For these patients, the major debate is whom to treat and with what. Some physicians prefer treatment when patients have very low platelet counts, whereas others prefer to "watch and wait" if there is no major ongoing bleeding.

Reduced quality of life is probably overlooked more often in this population than in adults because children do not express it as well as adults. It may be expressed as increased irritability, poor performance, or greater fatigue.

Serious bleeding is primarily intracranial hemorrage (ICH), which is estimated to occur in approximately 1 in 200–500 cases (Psaila et al. 2009). It may occur at presentation or at any time during the course of the disease. It is slightly over-represented in those with chronic disease.

Treatment effects are similar to those seen in adults. The major issue is that the patient can improve at any time up to the end of the first year so there is less need than with adults to provide a "definitive" therapy. Splenectomy is avoided whenever possible and is ideally reserved for those who have had ITP for more than 1 year, are older than 5 years, have very low platelet counts, bleeding, and are not doing well with other therapies. The effects of rituximab are similar to those seen in adults, with younger children not doing so well and adolescent girls within 1 year of ITP diagnosis responding best when rituximab is combined with dexamethasone (Chapin et al. 2016). TPO agents have recently been studied in children, with one small and three large randomized controlled studies completed (Bussel et al. 2011, 2015a, b; Tarantino et al. 2016; Grainger et al. 2015). All studies have shown good efficacy and good safety and resulted in eltrombopag being licensed for children with ITP down to the age of 1 by the FDA in the USA and by the EMA in Europe. Similar licensure is expected for romiplostim in the near future. More data demonstrating long-term safety and persistent efficacy is available for romiplostim than for eltrombopag at this time (Bussel et al. 2015a, b). One retrospective study has investigated bone marrow and not seen problems (e.g., high grades of fibrosis or abnormal cells) (Ramaswamy et al. 2014). An ongoing romiplostim study is prospectively exploring marrow findings.

Other treatments (e.g., immunosuppressive agents such as 6-MP and azathioprin) have also been studied in single-arm trials (Grace et al. 2012). In the 1980s, long-term IVIG was also tried but caused post-infusion headaches and was cumbersome because of the time needed for infusion. Similarly, intravenous anti-D was tried (Andrew et al. 1992) but its use declined with the discovery of serious intravascular hemolysis occurring for 1 in 1115 infusions (Gaines 2000). This was carefully reviewed after a "black box" warning was issued by the FDA (Despotovic et al. 2012).

Overall, the good news is that ITP in children mostly resolves spontaneously and the rate of serious bleeding is rare. The bad news is that it is not currently possible to predict the advent of chronic disease or of serious bleeding. Furthermore, chronic disease in children is not easier to treat than chronic disease in adults.

Conclusion

In summary, this is a very exciting time for ITP as newer treatments are better studied and additional treatments continue to be developed. Furthermore, other considerations are also being taken into account (e.g., HRQoL), but this is just one example of the "whole patient" approach. Molecular studies may allow separating patients into different groups and directing treatment choices; unfortunately this is still not possible.

We now know that, although there are many factors that influence megakaryocyte development and the regulation of platelet production, the role of TPO is key. It remains to be clarified whether aging platelets are cleared in the liver and stimulate synthesis of additional TPO, and what other factors are important and operative in this system to up- or downregulate endogenous TPO production. The more the autoimmune response is studied, the more complex it seems. The good news is that there are more targets for intervention; the bad news is that it is impossible to test them all. Although antibody-mediated platelet destruction (and impaired platelet production) is certainly central to the pathogenesis of ITP, the role of cell-mediated immunity has been studied on many levels and it seems that autoreactive T cells drive the disease in more difficult or more chronic ITP patients. Even though TPO receptor agonists are not the "be all and end all," if a patient responds to them they usually provide a good quality of life even if patients do not improve enough to be able to discontinue treatment. Because cure is the goal, perhaps additional rituximab combinations or better prediction of splenectomy outcome will allow us to take care of at least a sizeable fraction of our patients.

Take-Home Message Boxes

The diagnosis and management of ITP is still a work in progress. Although novel treatments have improved the management strategy in ITP, many questions remain to be answered and some patients continue to be refractory to treatment. A better understanding of the pathology of ITP, biomarkers to direct treatment and new therapeutics for refractory patients is needed.

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Fetal and Neonatal Alloimmune Thrombocytopenia

Gérald Bertrand and Cécile Kaplan

Abstract

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) results from the maternal immune response against fetal-specific antigens inherited from the father. FNAIT is the most common cause of severe fetal and neonatal thrombocytopenia in maternity wards. The most feared complication of FNAIT is the occurrence of intracranial hemorrhage. Since the first description of this condition in the 1950s, significant progress has been made in the understanding of the pathology and management of patients with alloimmune thrombocytopenia, but some questions remain unresolved. This chapter addresses the pathophysiology of FNAIT and discusses advances in the diagnosis and management of at-risk pregnancies.

Key Discoveries

- Progress in laboratory diagnosis has led up to now to the description of 28 platelet antigen systems involved in this pathology.
- Syncytiotrophoblastic microparticles expressing β_3 integrin spreading into the maternal blood circulation may be the cause of maternal immunization.
- Antenatal management is offered for subsequent pregnancies, preventing the occurrence of fetal/neonatal intracranial hemorrhages.
- With the development of noninvasive strategies for antenatal management, follow-up of the maternal alloantibody concentration during IVIG-treated pregnancies may be helpful for identifying therapy failure and managing delivery.
- Alternative therapies to maternal antenatal management with IVIG are under study, focusing on the transplacental passage of the maternal alloantibodies, the interaction with the fetal FcγRs, and prevention of immunization.

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Introduction

Thrombocytopenia, defined by a platelet count below 150×10^9/L, is the most common hematologic disorder observed in the fetus or neonate. Fetal/neonatal alloimmune thrombocytopenia (FNAIT) is the commonest cause of severe fetal (Hohlfeld et al. 1994) and neonatal thrombocytopenia in maternity wards (Dreyfus et al. 1997). It is considered to be the counterpart of hemolytic disease of the fetus and newborn (HDFN), but in contrast to HDFN, FNAIT frequently occurs during the first pregnancy. The most feared complication is the occurrence of intracranial hemorrhage (ICH) leading to death or neurologic sequelae. FNAIT, a potentially devastating disease, is not a rare event; its incidence has been estimated to be about 1/800 to 1/1200 live births in Caucasians.

Clinical Presentation

FNAIT is a diagnosis of exclusion after considering other causes of fetal or neonatal thrombocytopenia. Fetal thrombocytopenia has been defined as a platelet count $<150 \times 10^9$/L, irrespective of the gestational age. Fetal thrombocytopenia may be suspected in a variety of circumstances: e.g., congenital infection, chromosomal abnormalities, congenital/inherited

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platelet disorders (Wiskott-Aldrich syndrome), immune thrombocytopenia.

Unfortunately fetal alloimmune thrombocytopenia is more often discovered when ICH has occurred. Sonography reveals ventriculomegaly or fetal hydrocephalus. ICH has been documented regardless of implicated platelet alloantigens. With the development of imaging, ICH has been documented as early as 20 weeks gestation, and 80 % of ICH has occurred before 30 weeks gestation (Spencer and Burrows 2001). The deleterious consequences are death or neurologic sequelae.

It is very important to consider the onset of neonatal thrombocytopenia. Before 72 h of life, thrombocytopenia is almost always related to complications of pregnancy and/or delivery: placental insufficiency, hypoxia, perinatal infection, disseminated intravascular coagulation, and immune thrombocytopenia (alloimmune, autoimmune). After 72 h, it generally results from postnatal acquired bacterial infection or necrotizing enterocolitis.

Most commonly, neonatal alloimmune thrombocytopenia is suspected in an otherwise well-appearing, full-term infant with unexplained bruising and purpura at birth, or a few hours afterwards born to a pregnant healthy mother. In some circumstances, severe bleeding such as ICH, a large cephalohematoma, and gastrointestinal or genitourinary hemorrhage is observed. In any case, a platelet count should be obtained revealing isolated thrombocytopenia. Nonetheless, associations with other disorders do occur and should not be ignored (infection, chromosomal abnormality, maternal autoimmune thrombocytopenia). FNAIT may be suspected when the thrombocytopenia is more severe than anticipated for the diagnosis. A history of neonatal thrombocytopenia in prior pregnancies is also important to consider.

In the case of severe thrombocytopenia, the risks of life-threatening hemorrhage necessitate prompt diagnosis and effective therapy. It should be noted that neonatal thrombocytopenia may be asymptomatic when the platelet count is above 50×10^9/L and may be discovered by chance when performing a blood cell count for some other reason. The diagnosis is important for the management of index cases and for subsequent pregnancies. In all cases, raising the possibility of FNAIT should guide investigations accordingly.

Pathophysiology

FNAIT results from the production of maternal alloantibodies against fetal platelet antigens which are inherited from the father [human platelet antigen: HPA nomenclature (von dem Borne et al. 1995)].

The mechanisms of maternal immunization are partly understood. In contrast with HDN, platelet alloimmunization may occur during the first pregnancy (Bertrand et al. 2011). Findings have shown that fetal syncytiotrophoblasts express the integrin β_3 on their cell surfaces. Syncytiotrophoblastic microparticles spreading into the maternal circulation may possibly be the cause of immunization during pregnancy (Kumpel et al. 2008). The maternal immunogenic background is involved in HPA-1a alloimmunization. It has been shown that HPA-1a immunization is initiated by the binding of HPA-1a peptides to HLA-DRB3*01:01 and HLA-DRB4*01:01 molecules (Delbos et al. 2016; Loewenthal et al. 2013; Valentin et al. 1990). The immune response to the HPA-5b antigen has also been shown to be HLA associated, but studies of maternal immunization due to other HPA specificities are too small to draw conclusions in this regard.

The intensity of the thrombocytopenia may result from several factors:

- The active transfer of IgG through the placenta, from 13 weeks of gestation and the full expression of fetal platelet alloantigens as early as 18 weeks of gestation (Kaplan et al. 1985). This process depends on fetal Fc (FcRn) receptors. These receptors have various affinities according to IgG subclasses, due to several polymorphisms located on their genes (Einarsdottir et al. 2014).
- Adherence, phagocytosis and lysis of IgG-coated cells by IgG Fc receptors on macrophages may be modulated by glycosylation. Kapur et al. reported that anti-HPA IgG with a low amount of fucose displayed higher binding affinity to FcγRIIIa and FcγRIIIb, but not to FcγRIIa, as compared to antibodies with a high amount of Fc fucose. Moreover, the degree of anti-HPA-1a fucosylation has been correlated with the neonatal platelet count in FNAIT (Kapur et al. 2014).

Platelet Antigens Implicated in FNAIT

HPA Antigens

Platelet alloantigens are present on the platelet glycoproteins (GPs) GPIb-V-IX (von Willebrand receptor), $\alpha_{IIb}\beta_3$ complex (fibrinogen receptor), $\alpha_2\beta_1$ complex (collagen receptor), and CD109. Platelet antigens result from single amino acid substitutions. An international nomenclature was set up: HPA antigens are classified in the order of their discovery. Twenty eight HPA systems have been described up to now (Table 1). In each system, the most frequent allele is called "a," and the less frequent "b." Antigen frequencies vary among the populations studied so far.

Table 1 HPA nomenclature (human platelet antigen)

System	Antigen	Glycoprotein	Nucleotide change	Amino-acid change (mature protein)
HPA-1	HPA-1a HPA-1b	GPIIIa	176T>C	L33P
HPA-2	HPA-2a HPA-2b	GPIba	482C>T	T145M
HPA-3	HPA-3a HPA-3b	GPIIb	2621T>G	I843S
HPA-4	HPA-4a HPA-4b	GPIIIa	506G>A	R143Q
HPA-5	HPA-5a HPA-5b	GPIa	1600G>A	E505K
	HPA-6bw[a]	GPIIIa	1544G>A	R489Q
	HPA-7bw[a]	GPIIIa	1297C>G	P407A
	HPA-8bw[a]	GPIIIa	1984C>T	R636C
	HPA-9bw[a]	GPIIb	2602G>A	V837M
	HPA-10bw[a]	GPIIIa	263G>A	R62Q
	HPA-11bw[a]	GPIIIa	1976G>A	R633H
	HPA-12bw[a]	GPIbb	119G>A	G15E
	HPA-13bw[a]	GPIa	2483C>T	T799M
	HPA-14bw[a]	GPIIIa	1909_1911delAAG	K611del
HPA-15	HPA-15a HPA-15b	CD109	2108C>A	S682Y
	HPA-16bw[a]	GPIIIa	497C>T	T140I
	HPA-17bw[a]	GPIIIa	662C>T	T195M
	HPA-18bw[a]	GP1a	2235G>T	Q716H
	HPA-19bw[a]	GPIIIa	487A>C	K137Q
	HPA-20bw[a]	GPIIb	1949C>T	T619M
	HPA-21bw[a]	GPIIIa	1960G>A	E628K
	HPA-22bw[a]	GPIIb	584A>C	K164T
	HPA-23bw[a]	GPIIIa	1942C>T	R622W
	HPA-24bw[a]	GPIIb	1508G>A	S472N
	HPA-25bw[a]	GPIa	3347C>T	T1087M
	HPA-26bw[a]	GPIIIa	1818G>T	K580N
	HPA-27bw[a]	GPIIb	2614C>A	L841M
	HPA-28bw[a]	GPIIb	2311G>T	V740L

[a]w: Workshop, no antithetic antigen discovered

In persons of European origin, the HPA-1a antigen is responsible for the majority of cases of FNAIT [85 % cases of alloimmunizations (Davoren et al. 2004)], followed by HPA-5b (Kaplan et al. 1991) and -3a alloimmunizations (Glade-Bender et al. 2001) with a lower frequency. In East Asians, FNAIT is mainly linked to HPA-4b (Feng et al. 2006).

During recent years FNAIT has been reported involving rare or private antigens, most of them located in the $\alpha_{IIb}\beta_3$ complex. Recent studies have shown these low-frequency antigens as not being restricted to single families (Bertrand et al. 2007, 2013a; Kaplan et al. 2005). Consequently, during laboratory investigations, in the absence of fetomaternal incompatibility for the most frequent antigens according to ethnicity, a search for the presence of these low-frequency antigens is recommended.

The relevance of the anti-HPA-15a or HPA-15b alloantibodies in FNAIT requires further evaluation, due to technical difficulties to detect these antibodies.

HPA-2, located on platelet GPIbα, has not been considered as an important alloantigen implicated in FNAIT, since reported cases caused by this antigen are rare. It is currently unknown whether the paucity of reported cases of FNAIT mediated by HPA-2 is due to less immunogenicity of this antigen or due to a difference in pathology caused by HPA-2 antibodies. Recent data from a murine model suggest that antibodies against GPIbα may cause nonclassical signs of FNAIT [i.e., earlier spontaneous miscarriage but not neonatal bleeding (Li et al. 2011)], which may mask the severity and frequency of human anti-HPA-2-mediated FNAIT, thus reducing the observed number of cases.

It has been suggested that antibodies against GPIbα may cause platelet activation and thrombus formation in the placenta, which may impair placental function and lead to spontaneous miscarriage (Li et al. 2011).

Although platelet destruction is thought to be the major contributor to bleeding in affected patients, the effect of antiplatelet antibodies on vascular injury may affect bleeding and fetal growth. Several HPA antigens are expressed on the β3 integrin of the $\alpha_{IIb}\beta_3$ complex, which is also part of the integrin αvβ3 expressed on other cell types, such as angiogenic endothelial cells and invasive trophoblasts. It is therefore conceivable that maternal antibodies against HPA-1a may affect fetal angiogenesis, contributing to intrauterine growth restriction. Recently, reports suggest that impairment of angiogenesis, but not platelet or fibrin clots, may be the major cause of fetal embryonic hemorrhage (Yougbare et al. 2015).

HLA (Human Leukocyte Antigen) Molecules

Feto-maternal anti-HLA class I alloimmunization is common, as about one-third of multiparous women are sensitized to HLA class I. As platelets express both HLA-A and HLA-B antigens, antibodies may be responsible for thrombocytopenia. However, no systematic study has established a clear correlation between neonatal alloimmune thrombocytopenia and the presence of anti-HLA alloantibodies, except a few cases reported in the literature (Moncharmont et al. 2004; Thude et al. 2006).

Thrombocytopenia and Alloimmunization Anti-blood Groups Anti-A or Anti-B

In most individuals, platelets express only weakly A and B antigens of the ABO blood group on their surface. However, a low proportion of normal subjects unusually expresses strongly A and B antigens on the surface of their platelets. Few cases of anti-A or anti-B fetomaternal alloimmunizations have been published (Curtis et al. 2008).

Anti-GP Isoimmunizations

Isoantibodies occurring in mothers deficient in platelet CD36 [3–5 % of Asian or African ancestry (Ohto et al. 2004)] or deficient in the platelet $\alpha_{IIb}\beta_3$ complex (as observed in Glanzmann thrombasthenia) may also be involved in FNAIT (Curtis et al. 2002; Ohto et al. 2004; Leticee et al. 2005). The same situation may also be observed in patients suffering from Bernard-Soulier syndrome (absence of GPIb: Peitsidis et al. 2010).

Diagnosis and Therapeutic Strategies

When fetal ICH is suspected through imaging, it is important to establish the diagnosis of fetomaternal immunization (Fig. 1). Positive cases will be managed by close collaboration between obstetricians and neonatologists.

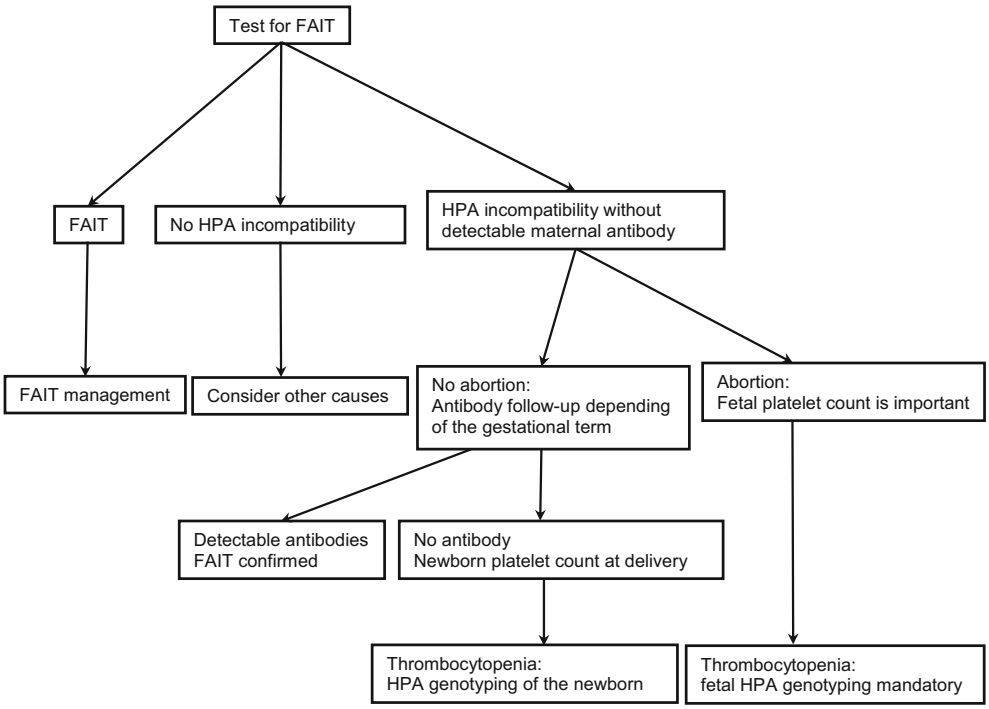

Fig. 1 Algorithm for laboratory investigations when a fetal alloimmune thrombocytopenia (FAIT) is suspected

Any severe or isolated neonatal thrombocytopenia should be explored.

The diagnosis of alloimmune thrombocytopenia will be ascertained only when the maternal alloantibody and the offending antigen present in the fetus or newborn are identified.

Investigations should be performed in a specialized laboratory that has the capacity to perform antigen-capture assays for antibody testing, and typing of common HPAs, and even rare or new platelet alloantigens.

Serology

The screening and identification of antiplatelet antibodies are currently performed by antigen-capture methods. The MAIPA (monoclonal antibody-specific immobilization of platelet antigens) is considered as the gold-standard reference method (Kiefel et al. 1987). This method is an antigen-capture assay, performed with two monoclonal antibodies (Mab), one targeting human IgG and the other targeting platelet glycoproteins. However, false-negative results might be observed resulting from competition between anti-GP Mab and human antibodies. The use of a panel of anti-GP Mab directed against the same GP with distinct epitopes may solve this problem. Maternal alloantibody detection is performed on the serum and tested (1) against a panel of group O donor platelets and (2) against the father's platelets. When alloimmunization involves a low-frequency platelet antigen, the MAIPA will only be positive with the paternal platelets.

Alternatively, the modified antigen-capture ELISA or MACE may be used (Fig. 2). In this technique, platelet GPs are precoated onto the microtiter plate, preventing disruption of the formed immune complexes during the lysis phase. Nevertheless, the conformational structure of the antigens may be modified.

The mixed passive hemagglutination, or MPHA, method bypasses the major pitfalls of the MAIPA and the MACE. However, antibody quantification cannot be done with this method, as the detection system is not colorimetric.

Some antibodies with a weak affinity are detected only by surface plasmon resonance (SPR) technology (Bakchoul et al. 2013; Peterson et al. 2013). Their clinical relevance remains to be determined.

Recently, the Luminex technology has been adapted to identify anti-HPA alloantibodies, which saves time and requires fewer biological samples (Porcelijn et al. 2014).

When a maternal alloantibody is not detectable despite parental antigenic incompatibility, it may be necessary to obtain new samples in the postnatal period and at the beginning of a new pregnancy.

Maternal autoimmunity should also be investigated; anti-GP autoantibodies may cross the placental barrier and cause fetal/neonatal thrombocytopenia, which is generally less severe when observed with alloantibodies. It has been reported that association with maternal alloimmunization is

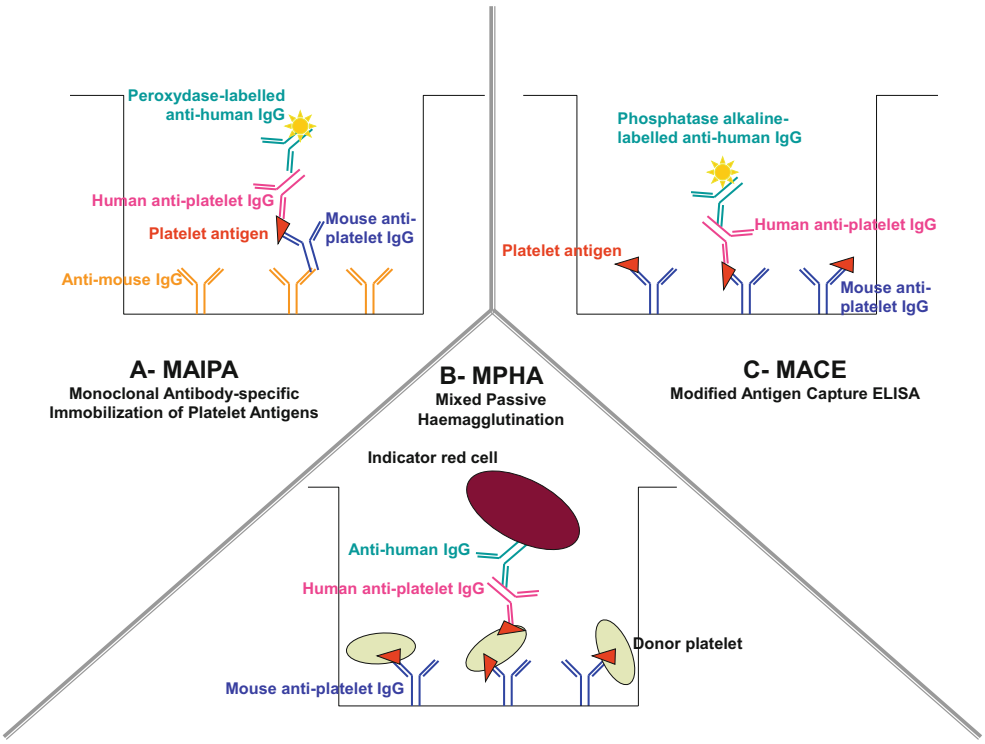

Fig. 2 Detection of antiplatelet antibodies using the MAIPA (*A*), MPHA (*B*), and MACE (*C*) methods. The final molecular architecture of antibodies and antigens is shown

not a rare event (Dreyfus et al. 1997). In that condition, thrombocytopenia can last for a longer time.

Platelet Typing

Platelet phenotyping is the best way to identify potentially immunogenic peptides. The main limitation for platelet phenotyping is the scarcity of specific, good-quality typing sera, especially for rare platelet antigens. Consequently, genotyping is the routine method for platelet typing. This approach obviates the need for patient platelets and suitable typing sera.

For appropriate counseling for subsequent pregnancies, the father's genotype should be performed whenever possible.

Unfortunately, genotype is not always representative of true phenotype. Discrepancies between phenotyping and genotyping may be observed when a genetic mutation hampers the technique itself [silent mutation located under one of the polymerase chain reaction primers, impairing DNA amplification (Bertrand et al. 2006a, 2010, 2013b; Conti et al. 2014; Nogues et al. 2013)].

Therapeutic Strategies

Postnatal Therapy

Therapeutic options will depend on the severity of thrombocytopenia.

In case of hemorrhage or a platelet count below 30×10^9/L during the first 24 h of life, the severely affected newborn should be promptly transfused with platelets, even without the results of laboratory investigations. Follow-up is important to maintain the platelet count above 50×10^9/L.

If the platelet count is above 30×10^9/L, usually no treatment is required in the absence of ICH.

For a premature infant, or in the case of an associated pathology, respiratory distress, or infection, platelet transfusion is required to maintain the platelet count above 50×10^9/L.

In any case, the platelet count should be monitored for at least 1 week to detect any fall in the platelet count.

Platelet Transfusion Therapy

In the context of FNAIT, the ideal platelets to be transfused are those that will not be destroyed by the maternal alloantibodies present in the newborn. Consequently, maternal platelets are the best product. They must be washed to remove the alloantibody and irradiated (to avoid graft versus host disease). However, due to logistical difficulties, maternal platelet transfusions are not often available.

To circumvent these problems, a national registry of HPA-typed donors has been developed in different countries (Allen et al. 2004); in addition frozen platelets may be used, as in France (Lee et al. 2002). However in an emergency, random platelet transfusion is an appropriate strategy pending the availability of compatible platelets later on (Kiefel et al. 2006). In order to limit the destruction of transfused random platelets, it is recommended to add IVIG (daily dose of 1 g/kg; 2 days). A recent report on a limited number of cases has shown that platelet transfusion alone was effective (Bakchoul et al. 2014). IVIG alone is not indicated as the sole therapy. It does not prevent hemorrhage; the lag-time efficacy is about 20 h.

Outcome

Cerebral imaging should be performed as part of a careful neurological examination before the newborn is discharged from hospital. A normal platelet count is usually obtained with or without treatment in 8–10 days, and in the absence of ICH, the outcome is favorable.

In case of ICH, death has been reported as affecting 10–15 % of the cases, or there are neurologic sequelae in 20 % of cases.

Management of Subsequent Pregnancies

Due to the recurrence of this condition in successive pregnancies with an incompatible fetus, usually implying an increasingly severe condition, antenatal therapy has been offered since 1984 (Daffos et al. 1984; Kaplan et al. 1988; Bussel et al. 1988).

The important questions are the evaluation of the fetal status, identification of fetuses requiring therapy, and monitoring the response to therapy.

Antenatal therapy nowadays is offered to women with a past history of an affected infant.

The pregnant women should be monitored in referral centers offering genetic counseling.

The determination of the fetal status is a prerequisite. In the case of paternal heterozygosity for the offending antigen or an unidentified father, fetal platelet genotyping is necessary. This may be performed on amniotic cells. However, amniotic liquid puncture is an invasive method with adverse side effects, premature labor, or even in utero death. New developments in fetal DNA isolated from maternal plasma are promising. A protocol is already in use for antenatal diagnosis of HPA-1 incompatibility (Le et al. 2013).

Due to ethical reasons, randomized controlled trials on the efficacy of different protocols have not been done. There is a general consensus on maternal therapy with IVIG with or without steroids as a first-line treatment while minimizing invasive procedures. Therefore, fetal blood sampling is becoming less frequent. Delivery by elective C-section is usually proposed when the fetal status is unknown. Compatible platelets are prepared to be available if necessary for transfusion immediately after birth.

Dosing and timing of maternal therapy depend on the strategy in use by the team, and protocols may differ. Therapy stratification depending on the sibling status, with or without ICH, has been advocated (Bussel et al. 2010). Between 1992 and 2015, 16 retrospective studies on IVIG antenatal therapy were published (582 newborns). Only 13 cases of ICH were reported (2.2 %).

In our experience for the standard risk, i.e., a sibling without ICH, we recommend IVIG 1 g/kg/week, from 20 to 22 weeks of gestation until delivery by C-section at 39 weeks of gestation (Bertrand et al. 2011). In the case of high-risk pregnancy, siblings with ICH, the therapy will start at 16–18 weeks of gestation. With this strategy, no ICH was observed in our retrospective study on 75 HPA-1bb pregnant women treated with IVIG. Addition of steroids (prednisone 0.5 mg/kg/day) from 30 to 32 weeks of gestation significantly increased the mean newborn platelet count: 135×10^9/L (54 newborns) with IVIG and steroids versus 89×10^9/L (27 newborns) with IVIG only (Bertrand et al. 2011).

For the most severely affected fetuses, recent studies have suggested that IVIG $\times$ 2 infusions of 1 g/kg/week combined with 0.5–1.0 mg/kg/day prednisone is the most effective medical regimen (Vinograd and Bussel 2010).

No side effect of maternal therapy with IVIG has been reported in the children on long-term follow-up (Radder et al. 2004).

In the absence of fetal blood sampling to assess the fetal platelet count, searching for maternal parameters predictive of therapy effectiveness is challenging. We have shown that the maternal anti-HPA-1a alloantibody concentration is a predictive variable for severe fetal thrombocytopenia when the test is performed early in the pregnancy and before any treatment (Bertrand et al. 2006b). Follow-up of the antibody concentration during IVIG-treated pregnancies appears to be helpful for identifying therapy failure and managing delivery (Bertrand et al. 2014; Killie et al. 2007a; Sainio et al. 2013).

The sisters of the homozygous women who gave birth to an affected infant should be aware, and counseling offered to detect high-risk pregnancies.

Other Therapeutic Approaches

Alternative therapies to maternal IVIG are focused on the transplacental passage of the maternal alloantibodies, the interaction with the fetal FcγRs, and prevention of immunization.

Anti-FcγR immunoglobulins may have the capacity to reduce platelet destruction (Li et al. 2011). Another approach in preventing platelet destruction is the use of recombinant HPA-1a-specific antibodies with abrogated Fcγ receptor binding (Ghevaert et al. 2013). These antibodies have the capacity to saturate available HPA-1a-binding sites from fetal platelets without activating FcγR signaling. These strategies have been studied using murine FNAIT models. Due to physiological differences between mouse and human placentation, transposition to human FNAIT therapy is still under discussion.

Very recently, Ni and colleagues suggested a new treatment option for thrombocytopenia, based on sialidase inhibition (Jansen et al. 2015). This option relies on the observation that platelets normally gradually loose surface sialic acid as they circulate and age through cleavage by sialidases (Grozovsky et al. 2015). It has been shown in vitro that desialylation of the platelet von Willebrand factor receptor complex triggers platelet destruction, which could be circumvented with a sialidase inhibitor. In vivo, they performed a retrospective observational study on patients treated with oseltamivir (sialidase inhibitor) and found a significant increase in platelet count in treated patients, proof of the principle that sialidase inhibition increases platelet count in humans, probably by decreasing clearance of platelets (Jansen et al. 2015).

A study in the Norwegian population suggests the possibility of a prophylactic treatment similar to the prevention of HDN (Kjeldsen-Kragh et al. 2012). This relies on the hypothesis that in the majority of cases, maternal immunization occurs at the first delivery and not during pregnancy, despite reports showing immunization during the first pregnancy (Mueller-Eckhardt et al. 1989; Durand-Zaleski et al. 1996; Bertrand et al. 2011). To examine the validity of the hypothesis, a proof-of-concept study on a murine model has been reported (Tiller et al. 2012).

Cost-Effectiveness of a Screening Strategy of High-Risk Pregnancies

As the first pregnancy may be affected by FNAIT, screening at-risk pregnancies for prevention of deleterious consequences is of the utmost interest. This strategy is still a subject of debate, despite studies from European countries showing that the implementation of such screening is cost-effective given the damage caused by ICH (Killie et al. 2007b). Several screening strategies have been reported in the literature, screening pregnant women or newborns and counseling for the next pregnancy (Durand-Zaleski et al. 1996).

The main issues are:

- The detection of maternal alloantibodies with the potential difficulties as mentioned above.
- The evaluation of the fetal risk during the pregnancy under consideration, without invasive procedure. Quantification of alloantibodies (concentration below a threshold) may be considered, but a gray zone does exist.
- The protocol to be proposed for antenatal management, especially in the case of first pregnancies.

Conclusion

In conclusion, FNAIT first described in the early 1950s is still a matter of interest for immunologists, obstetricians, and neonatologists. Progress has been made in diagnosis and management for a better quality of life. However, larger collaborative studies are still needed for improvement in diagnosis and for specific therapies.

Take Home Messages

- Fetal and neonatal alloimmune thrombocytopenia is not a rare event: Incidence of 1/800 to 1/1000 live births in persons of European origin.
- Any severe isolated fetal or neonatal thrombocytopenia should be explored.
- Laboratory diagnosis relies on the identification of maternal antiplatelet alloantibodies directed against fetal platelet antigens inherited from the father.
- The most feared complication is intracranial hemorrhage resulting in death or long-term disability.
- In emergency, in case of severe neonatal thrombocytopenia or hemorrhage, platelet transfusion therapy should be done promptly.
- Management of subsequent high-risk pregnancy is offered in referral centers due to recurrence for incompatible fetuses.

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Drug-Induced Thrombocytopenia: Pathogenesis, Diagnosis and Management

Beng H. Chong and James J.-H. Chong

Abstract

A large number of drugs have been reported to cause immune thrombocytopenia. Recent research has considerably increased our knowledge of the cellular mechanisms that drive this immune reaction to drugs. The diagnosis is usually made clinically after considering the causal relationship with the suspected drug and the occurrence of the thrombocytopenia. The mainstay of treatment is essentially cessation of the suspected drug or drugs. The platelet count usually returns to normal limits within 1–2 weeks. However, high-dose glucocorticosteroid and IVIg are often administered because of the high risk of bleeding associated with severe thrombocytopenia. This review will focus on the various cellular mechanisms that cause drug-induced immune thrombocytopenia and will also discuss its diagnosis and management.

Introduction

Hippocrates used the term "purpura" to describe the purple spots on the skin of patients with pestilential fever without knowing its association with bleeding or thrombocytopenia (Jones and Tocantins 1933). "Purpura" comes from the Latin word "'*ρэrpэrə*" meaning purple. In the sixteenth century, Lusitanus observed that purpura could occur in the absence of fever. Three centuries later, Krauss in 1883 and Denys in 1887 recognized "purpura" as bleeding under the skin, "purpura haemorrhagica" caused by a decrease of blood platelets (Jones and Tocantins 1933). In 1883, Vipan described the association of purpura with quinine ingestion (Vipan 1865). In 1928, Rosenthal provided the first evidence that drug ingestion could lead to thrombocytopenia by showing prompt decrease in platelets when a patient with quinine-induced thrombocytopenia was re-challenged with quinine (Leach et al. 1997).

Many drugs have been implicated to cause thrombocytopenia (Chong 1991; Aster et al. 2009; Aster and Bougie 2007; George et al. 2006; Chong et al. 2013). Drug-induced thrombocytopenia is common because it is caused by a large number of drugs, many of which are commonly used medications (see below). However, it is difficult to diagnose and is often unsuspected and under-recognized. Yet it is potentially serious as it can cause severe bleeding and even fatal haemorrhage such as an intracranial bleed (Freiman 1990; Fireman et al. 1981; Samaranayake and Yap 2014).

Mechanisms of Drug-Induced Thrombocytopenia

Although drug-induced thrombocytopenia has been identified for more than a century, it is only in recent years that its mechanisms have been characterized, and even now

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some aspects of its pathophysiology remain unclear. Drugs cause thrombocytopenia by two broad mechanisms:

(a) Suppression of platelet production (non-immune and immune)
(b) Increased platelet clearance (non-immune and immune)

Mechanisms of Non-immune Drug-Induced Thrombocytopenia

(a) *Suppression of platelet production (non-immune)*. Drugs involved in this mechanism are usually antineoplastic/cytotoxic drugs such as alkylating agents (cyclophosphamide, adriamycin, melphalan) and antimetabolites (methotrexate) which cause predictable, dose-dependent myelosuppression leading to pancytopenia. However, some non-cytotoxic drugs also suppress platelet production, but they usually cause isolated mild but sometimes severe thrombocytopenia. These drugs include histone deacetylase inhibitors (HDIs) such as panobinostat (used in treatment of cancer) (Iancu-Rubin et al. 2012; Matuoka et al. 2007; Bishton et al. 2011) and valproate (used as an anticonvulsive and in psychiatry treatment) (Eastham and Jancar 1980; Kaufman and Gerner 1998; Trannel et al. 2001; Gesundheit et al. 2002; Proulle et al. 2000; Sleiman et al. 2000), anagrelide (used in treatment of essential thrombocythaemia) (Wang et al. 2005; McCune et al. 1997), linezolid (an oxazolidinone antibiotic used in treatment of drug-resistant bacterial infection) (Attassi et al. 2002; Orrick et al. 2002; Minson and Gentry 2010; Matsumoto et al. 2010; Pascoalinho et al. 2011; Cossu et al. 2014), interferon-α (used in hepatitis B treatment) (Li et al. 2010; Sata et al. 1997), imatinib mesylate (a -tyrosine-kinase inhibitor used in treatment of chronic myeloid leukaemia) (Sashida et al. 2003; Rajappa et al. 2007) and bortezomib (a proteasome inhibitor used in multiple myeloma treatment) (Lonial et al. 2005). HDIs suppress megakaryocyte maturation by inhibiting proplatelet formation and platelet release from megakaryocytes (Iancu-Rubin et al. 2012; Bishton et al. 2011). With regard to other drugs, the mechanism whereby they inhibit megakaryocytes is still unclear. With valproate, linezolid and imatinib, if the thrombocytopenia is profound, there may be an additional reason such as an immune mechanism (Proulle et al. 2000; Pascoalinho et al. 2011; Rajappa et al. 2007) or renal dysfunction (Matsumoto et al. 2010; Cossu et al. 2014).

(b) *Increased platelet clearance (non-immune)*. Drugs can increase platelet clearance by either an immune or non-immune mechanism. Drugs that induce thrombocytopenia by a non-immune mechanism include interleukin-2, growth factors such as granulocyte (G)-monocyte (M) colony stimulating factor (CSF), G-CSF and M-CSF (Nash et al. 1995; Minelli et al. 2009; Baker and Levin 1998), cytokines such as tumour necrosis factor (TNF)-α/interferon (IFN)-γ (Michelmann et al. 1997) and amrinone (Kinney et al. 1983; Ross et al. 2009). The mechanism whereby these drugs cause thrombocytopenia is still largely unknown, but the growth factors are believed to induce thrombocytopenia by macrophage activation (Nash et al. 1995; Baker and Levin, 1998) and TNF-α and IFN-γ by endothelial activation (Michelmann et al. 1997).

Drug-Induced Thrombotic Thrombocytopenic Purpura (TTP)

Drugs can also cause TTP, but the mechanism for the thrombocytopenia is different from that of the "conventional" drug-induced immune thrombocytopenia (DITP), a term usually reserved for thrombocytopenia caused by drug-dependent antibodies directed against platelets and/or megakaryocytes.

In a systemic review of published reports before March 2014, 78 drugs have been implicated to cause TTP. Only 22 drugs had evidence of a definite causal relationship with TTP (Al-Nouri et al. 2015). Of these, three drugs (quinine, cyclosporine and tacrolimus) accounted for the majority of the patient reports with definite evidence. On the other hand, the Food and Drug Administration (FDA) safety databases reveal that ticlopidine and clopidogrel (thienpyridine-derived anti-platelet agents) are the two most common drugs associated with TTP (Zakrija et al. 2009). Other drugs with definite evidence of association with TTP are bevacizumab, cocaine, docetaxel, everolimus, gemcitabine, interferon-α, interferon-β, mitomycin, muromonab-CD3, oxaliplatin, penicillin, pentostatin, quetiapine, sirolimus, sulfisoxazole, sunitinib, tacrolimus, trielina and vancomycin and vincristine (Al-Nouri et al. 2015).

The mechanisms whereby drugs induce TTP are largely unknown. The microvascular endothelial cell (EC) injury may be caused by the direct toxic effect of the drug itself (e.g. cyclosporine and tacrolimus) or a drug-related autoantibody (e.g. quinine-, oxaliplatin- and vancomycin-induced TTP) (Reese et al. 2015; Al-Nouri et al. 2015). EC damage leads to deficiency of ADAMTS13 which may be exacerbated by anti-ADAMTS13 autoantibodies such as in thienopyridine-associated TTP (Zakrija et al. 2009). EC injury also induces the release of extra-large von Willebrand factor multimers which cause platelet aggregation and microvascular thrombosis which in turn induces microangiopathic haemolysis and end-organ damage (e.g. renal impairment). In thienopyridine-associated TTP,

Zakrija et al. (2009)) revealed that the majority of patients have ADAMTS13 autoantibodies and severe thrombocytopenia, and they response well to therapeutic plasma exchange, but the minority have severe renal impairment and direct EC injury by drug, and they respond less well to plasma exchange. TTP will not be further discussed in this chapter as it is out of the scope of this review.

This review will focus on DITP which is mediated by drug-dependent platelet antibodies. Thrombocytopenias due to myelosuppression, megakaryocyte suppression and accelerated platelet clearance by non-immune mechanisms will also not be discussed further in this chapter. Heparin-induced thrombocytopenia (HIT) which has distinctly different clinical features, mechanism, diagnostic approach and treatment (Chong and Isaacs 2009) will be discussed in another chapter.

Drug-Induced Immune Thrombocytopenia (DITP)

Drugs that Cause DITP

Reese et al. (2010) investigated 1468 drugs suspected of causing DITP using three sources, namely, (1) previous case reports (253 drugs); (2) laboratory data from the Blood Centre of Wisconsin, USA (202 drugs); and (3) the Adverse Event Reporting System (AERS) of the US Food and Drug Administration (1444 drugs). However, only 102 drugs were listed in all three sources and these drugs probably represent the drugs most commonly suspected to cause DITP. Of these 102 drugs, 24 had definitive evidence that they cause DITP, namely, clinical data showing a causal relationship and the detection of drug-dependent anti-platelet antibodies. Of these 24, 12 drugs had drug-dependent antibodies identified in ten or more patients, providing strong and definitive evidence of DITP. These 12 drugs are commonly used in clinical practice such as antibiotics/antituberculotics (ampicillin, piperacillin, rifampicin, ethambutol, sulfisoxazole, trimethoprim/sulfamethoxazole and vancomycin), anti-platelets/$\alpha_{IIb}\beta_3$ inhibitors (abciximab, eptifibatide, tirofiban), non-steroidal anti-inflammatory agents (ibuprofen, naproxen), anticonvulsants (carbamazepine, phenytoin, valproic acid) and quinine/quinidine. Arnold et al. (2013) compiled a similar list of 36 drugs which satisfied all clinical criteria of DITP plus detection of drug-dependent antibodies, but only 16 of these drugs had positive test results by two of more laboratories. There are 24–36 drugs that satisfy clinical and laboratory criteria for DITP reported by multiple sources but 12–16 drugs with very strong evidence of DITP (Reese et al. 2010; Arnold et al. 2013). With time, more and stronger evidence will emerge for more drugs, and the list of drugs with definite/strong evidence of DITP will increase.

In addition, "DITP" may also occur with ingestion of beverages (e.g. tonic water which contains quinine) (Korbitz and Eisner 1973; Brasic 2001), herbal medicines (e.g. *Jui*, a Chinese herbal tea) (Azuno et al. 1999; Ohmori et al. 2004) and certain foods (e.g. *tahini*, pulped sesame seeds) (Arnold et al. 1998).

In some instances, DITP occurs in the presence of two drugs. This may happen when two concomitant drugs each induce an immune response and produce a drug-specific anti-platelet antibody. Alternatively, a drug increases the blood concentration of another drug or its metabolite(s). An example of the former is a patient, receiving both piperacillin and vancomycin, who developed a piperacillin- and vancomycin-dependent antibody resulting in severe thrombocytopenia which resolved only when both drugs were withdrawn (Anand and Chauhan 2011). An example of the latter is dexamethasone and phenytoin (Pandey and Yarlagadda 2012). Dexamethasone inhibits epoxide hydralase increasing the concentration of phenytoin epoxide which is responsible for the thrombocytopenia. Awareness of this drug interaction may help to prevent DITP with these drugs.

Incidence of DITP

The incidence of DITP varies with the drug implicated. Studies based mainly on national surveillance and hospital records reported incidences between 0.6 and 1.8 per 100,000 (Bottiger and Westerholm 1972; Bottiger and Bottiger 1981; Pedersen-Bjergaard et al. 1996, 1997, 1998; Danielson et al. 1984). For drugs such as quinine and trimethoprim/sulfamethoxazole, the reported incidence is higher, 36 and 28 per 1,000,000 per week of drug exposure, respectively (Kaufman et al. 1993). These studies may have underestimated the true incidences as diagnoses of DITP are difficult (George et al. 2006; George and Aster 2009). However, the frequencies with drugs such as heparin and $\alpha_{IIb}\beta_3$ inhibitors are higher. As many as 1–2 % of patients develop thrombocytopenia with their first exposure to $\alpha_{IIb}\beta_3$ inhibitors (abciximab, tirofiban and eptifibatide) and 10–12 % on re-exposure to abciximab (Curtis 2014). About 0.5–5 % of patients receiving unfractionated heparin develop thrombocytopenia (Shantsila et al. 2009), with a lower frequency with low molecular weight heparin, ~0.2 %.

Pathophysiology of DITP

As in non-immune drug-induced thrombocytopenia, the drug-dependent antibodies and drugs cause thrombocytopenia by cellular processes that (1) increase platelet clearance and/or (2) suppress platelet production. Before these

processes can occur, the drug-dependent antibodies and the inducing drugs will have to bind to platelets and/or megakaryocytes. The mechanisms whereby the antibodies and drugs interact with platelets/megakaryocytes will be discussed first.

How Does the Drug-Dependent Antibody Bind to Platelets in the Presence of the Drug?

DITP is mediated by a drug-dependent antibody (usually IgG) that binds to platelets and/or megakaryocytes in the presence of the sensitizing drug. Several mechanisms for the antibody-platelet-drug or antibody-megakaryocyte-drug interactions have been proposed. These proposed mechanisms will be discussed below:

1. *Hapten mechanism*. This was the mechanism believed to cause all DITP in the 1940s based mainly on evidence from animal studies (Ackroyd 1953; Shuman 1958, 1964). Drugs were thought to be too small by themselves to become immunogenic. It was postulated that they covalently bind to a macromolecule such as a protein and act as haptens to elicit an immune response inducing the formation of hapten-dependent antibodies. These antibodies recognize the macromolecule only when the hapten is attached. Subsequently, studies of patients with DITP show no evidence of drugs acting as haptens except perhaps penicillin and its derivatives (Garratty and Petz 1975; Murphy et al. 1983).
   Penicillin if administered to patients in large doses can covalently bind to proteins on red cell surface via its reactive β-lactam ring. Penicillin acting as a hapten can induce the generation of hapten-dependent antibodies which induce destruction of red cells causing autoimmune haemolytic anaemia (Garratty and Petz 1975). A recent study reported that penicillin derivative piperacillin can also induce the formation of hapten-dependent antibodies that target piperacillin-coated red cells and may possibly cause piperacillin-induced autoimmune haemolytic anaemia (Garratty 1993). It has been postulated that the hapten mechanism may also account for the thrombocytopenia occasionally seen in patients receiving penicillin and piperacillin (Murphy et al. 1983). However there is no convincing experimental evidence to support this hypothesis.
2. "*Quinine-type*" *immune thrombocytopenia, the prototype DITP*. The mechanism whereby quinine or its isomer (quinidine) induces thrombocytopenia has been extensively studied. Most other drugs cause immune thrombocytopenia by a similar or slightly modified mechanism. In the 1940s–1980s, it was believed that quinine, quinidine and most drugs induced thrombocytopenia by the hapten mechanism (Fig. 2a). The drug-dependent antibody reacts with quinine (attached to a plasma protein [macromolecule]), and the antibody-antigen complex then binds via Fc domain to platelet which is then destroyed as "an innocent bystander" as the antibody has no specificity for platelets ("innocent-bystander mechanism").
   (a) *Target antigen*. In 1983, we provided the first evidence that the drug-dependent antibody in a patient with quinidine-induced thrombocytopenia recognized a specific platelet surface protein. We immunoprecipitated GPIb-IX complex from platelet lysate with the patient's quinidine-dependent antibody using staphylococcal protein A (Chong et al. 1983). This observation not only indicates that the platelet antigen is GPIb-IX complex but it also suggests indirectly that the antibody binds to platelets via its Fab domain and not its Fc domain. This was a radical finding that challenged the dogma of that time, i.e. the "innocent-bystander" mechanism (Shuman 1958, 1964). This finding led subsequently to studies which directly showed that drug-dependent antibodies bind to platelets with their Fab domain in the presence of drugs (Smith et al. 1987; Christie et al. 1985). Later, another platelet drug-related antigen, $\alpha_{IIb}\beta_3$, was also identified (Chong et al. 1991; Visentin et al. 1991). $\alpha_{IIb}\beta_3$ complex and/or GPIb-IX complex are now known to be target antigens in DITP caused not only by quinine/quinidine but also most other drugs (Gentilini et al. 1998; Burgess et al. 1998, 2000; Asvadi et al. 2001; Peterson et al. 2008). One report suggested PECAM-1 as the target antigen for patients with thrombocytopenia induced by carbimazole (Kroll et al. 2000).
   In 2015, there is debate as to the exact mechanism by which the quinine-dependent antibody binds to platelets in the presence of the drug despite our knowledge of the precise platelet location of the antibody binding sites. There are at least two proposed hypotheses and they are discussed below:
   - *Hybrid paratope mechanism*. Recently, Zhu et al. (2015) and Bougie et al. (2015) proposed a *hybrid paratope* mechanism of drug-dependent antibody binding to the platelet antigen. Their hypothesis was based on the findings of their studies of two quinine-dependent monoclonal antibodies (moAbs), 314.1 and 314.3, which resembled human quinine-dependent antibodies. These moAbs recognized an epitope on platelet GPIIbβ and bound to the target epitope with low affinity in the absence of quinine. They found that quinine did not bind GPIIbβ but instead bound with high affinity and specificity the Fab domain of the

moAbs, 314.1 and 314.3, in a ratio of roughly 2:1 (quinine to moAb). These investigators also showed by crystallography strong drug binding to the complementarity-determining regions (CDRs) of the antibodies. Drug binding induced conformational changes in the CDRs resulting in the moAbs binding with enhanced affinity to GPIIbβ (Fig. 1a). *Hybrid paratope* was suggested as an appropriate name for their proposed mechanism. This is because (1) quinine combines with the CDR loop of the antibody to form *a paratope* which then binds to the epitope on platelet GPIIbβ, and (2) the paratope is a hybrid comprising the antibody CDR loop and quinine (Zhu et al. 2015). The *hybrid paratope* is an unconventional but novel mechanism. As the hypothesis is a departure from the conventional concept of drug sensitization, a relevant question is how does the patient become sensitive to quinine? These investigators suggested that quinine fortuitously binds to the B cell receptor and thus induces the production of quinine-dependent antibodies (Zhu et al. 2015).

However, there are additional unanswered questions: (1) Do the moAbs, 314.1 and 314.3, resemble exactly the quinine-dependent antibodies of patients with DITP? For example, does quinine also bind with high affinity to the CDR of the human drug-dependent antibodies? (2) Is the *hybrid paratope* mechanism also applicable to the human quinine-dependent antibodies that recognize other platelet antigens such as GPIIIa, GPIX and GPIbα? (3) How does the specific B

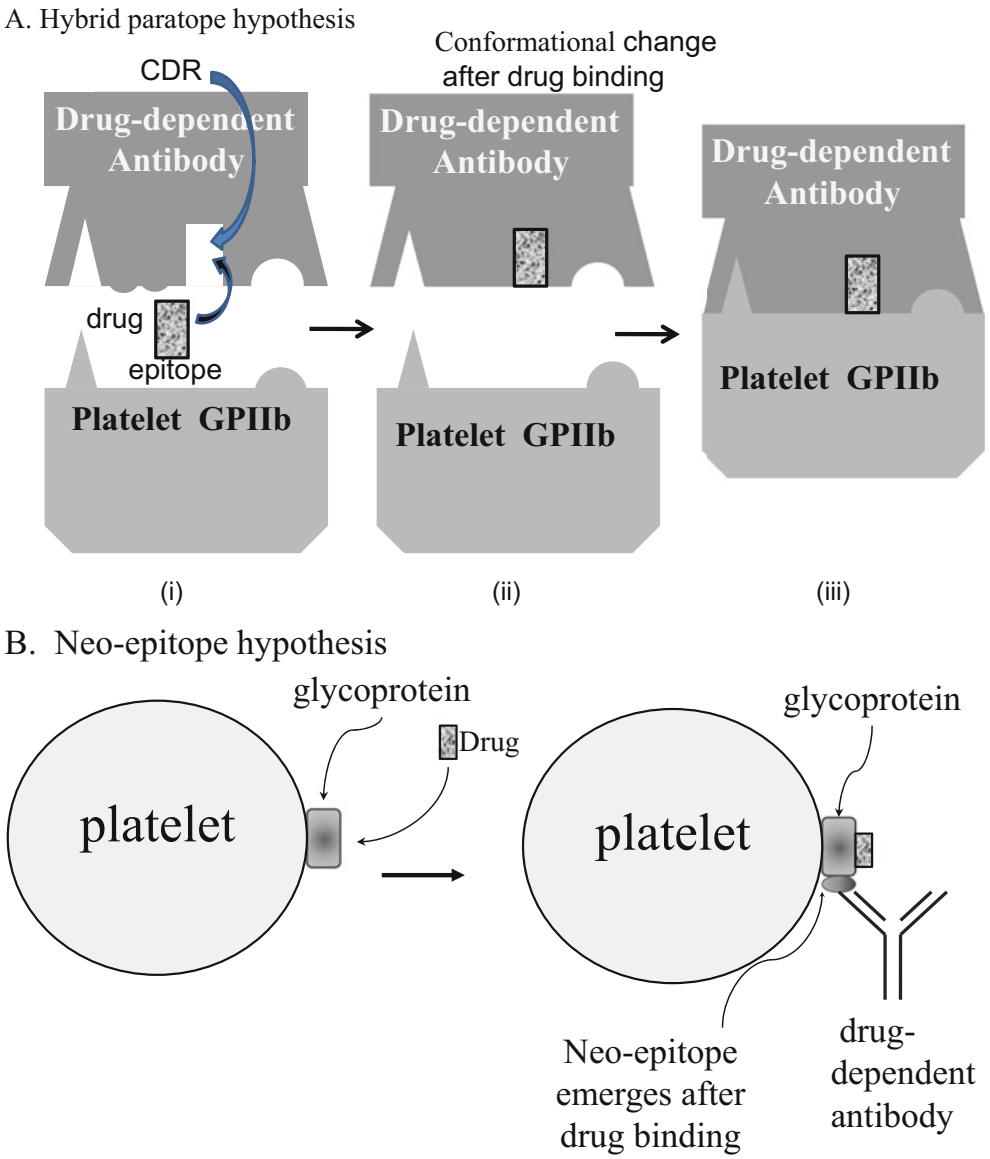

Fig. 1 Quinine-type drug-induced thrombocytopenia—hypotheses of antibody-drug-glycoprotein interaction. (**a**) *Hybrid paratope hypothesis*: Drug binds to the CDR of the drug-dependent antibody which has rough specificity and binds with low affinity to the target antigen, GPIIb, in the absence of the drug. Drug binding induces a conformational change in the CDR such that the antibody now has high specificity for and binds with high affinity to GPIIb. (**b**) *Neoepitope hypothesis*: The drug binds to target glycoprotein (antigen), GPIX or GPIIIa, inducing a conformational change such that a neoepitope, otherwise cryptic, is exposed so that the drug-dependent antibody can bind with high specificity and affinity

cell that recognizes quinine come into existence since quinine is too small a molecule to be antigenic? Do they occur in response to the weak non-covalent binding of quinine to the target antigen (GPIIbβ)?

- *The neoepitope hypothesis*. The conventional concept of drug sensitization is that the drug binds to an autologous protein, usually non-covalently, and induces a conformational change in the protein giving rise to a neoepitope that is previously unseen by the immune system. This leads to the production of an antibody that binds drug dependently to the neoepitope (Fig. 1b). In the case of quinine-induced thrombocytopenia, the autologous protein is GPIb, GPIX or GPIIIa. The antibody-binding domain has been fine mapped to the membrane-proximal ectodomain of GPIX (R110–Q115) (Asvadi et al. 2001), the N-terminal domain of GPIbα (residues 283–293) (Burgess et al. 1998) and the hybrid and plexin/semaphorin/integrin (PSI) domains of GPIIIa (A50–D66) (Peterson et al. 2008). GPIX (R110–Q115) and GPIIIa (A50–D66) are located in the regions of the platelet receptors that are highly hyperflexible (Xiong et al. 2001; Zhu et al. 2008; Mo et al. 2009; Dill 1991). Li (2010) suggested that as a result of the protein hyperflexibility, the drug binds to and stabilizes different non-native conformations of the receptors; some are recognized as foreign and induce an immune response (Li 2010). Heterogeneous drug-dependent antibodies are thus generated, and they recognize heterogeneous epitopes that cluster around a specific site of the platelet receptor. Hyperflexibility of GPIX domain, R110–Q115, may also explain why it is the binding site of at least four human drug-induced antibodies, namely, quinine-, quinidine-, rifampicin- and ranitidine-dependent antibodies (Asvadi et al. 2001; Burgess et al. 1998; Peterson et al. 2008). Hyperflexibility of the protein may allow drugs of different chemical structures (quinine, quinidine, rifampicin and ranitidine) to bind to this domain of GPIX (Li 2010).

  One weakness of this hypothesis is that the drug binds only weakly and non-covalently to the platelet receptor. As Li (2010) explained above, non-covalent drug binding to a hyperflexible protein may still permit induction of an immune response.

3. *Thrombocytopenia induced by $\alpha_{IIb}\beta_3$ inhibitors* (*fibans*). Fibans (tirofiban and eptifibatide) are synthetic small molecules that bind tightly to the arginine-glycine-aspartic acid (RGD) recognition site on $\alpha_{IIb}\beta_3$. They block fibrinogen binding, platelet aggregation and platelet thrombus formation (Topol et al. 1999). The fibans are used to treat acute coronary syndrome and to prevent stent thrombosis after percutaneous transluminal coronary angioplasty and stenting (Topol et al. 1999). Clinical trials (The RESTORE Investigators 1997; The PURSUIT Trial Investigators 1998) and subsequent clinical experience revealed that 0.1–2.0 % of patients who received fibans developed severe thrombocytopenia within 24 h of the first drug exposure (Patel et al. 2005; Coons et al. 2003; Rezkalla et al. 2003; Billheimer et al. 2002; Bougie et al. 2002; Seiffert et al. 2003). The incidence of severe thrombocytopenia in large clinical trials was lower than that observed in smaller studies. In the large trials, the thrombocytopenia might have been under-recognized. In contrast, smaller studies often included selected patient populations and hence there is a tendency to overestimate the incidence of thrombocytopenia.

   Thrombocytopenia is mediated by drug-dependent antibodies against $\alpha_{IIb}\beta_3$ that can occur naturally. This may explain the rapid onset of the thrombocytopenia (Bougie et al. 2002). These antibodies have also been detected in healthy individuals with no previous drug exposure (Aster et al. 2009; Aster and Bougie 2007; Bougie et al. 2002). Previous research has shown that RGD peptide can induce changes in $\alpha_{IIb}\beta_3$ resulting in the emergence of cryptic epitopes which are recognized by certain moAbs (Frelinger et al. 1991; Kouns et al. 1991; Jennings et al. 2000). These epitopes are known as "ligand-induced binding sites (LIBS)". The LIBS were expected to be also the epitopes of the fiban-dependent antibodies, but a recent study by Bougie et al. (2012) showed conclusively that they are not. They used the term MIBS (mimetic-induced binding sites) to denote the domains that emerge after fiban binding to $\alpha_{IIb}\beta_3$ (Fig. 2c). MIBS were located in the head region of $\alpha_{IIb}\beta_3$, near the RGD recognition site, probably on the β-propeller domain of GPIIb or the βA domain of GPIIIa (Bougie et al. 2012). The fiban-dependent antibodies were specific to the inducing drug (tirofiban or eptifibatide) in the majority of patients, but in the minority the antibodies cross-reacted with other fibans (Bougie et al. 2012; Chong 2012).

4. *Abciximab-induced thrombocytopenia*. Abciximab is a chimeric (mouse/human) monoclonal antibody (Fab fragment) that binds to the βA domain of GPIIIa and inhibits fibrinogen binding and thrombus formation. About 2 % of patients receiving the drug suffer acute, severe thrombocytopenia within 2–48 h within the first exposure to the drug (Kilickiran et al. 2008; Jubelirer et al. 1999; Curtis et al. 2002, 2004). The incidence is increased to 12 % when the drug is given again within 30 days (Bougie et al. 2012; Mascelli et al. 1998). In a subgroup of patients, the

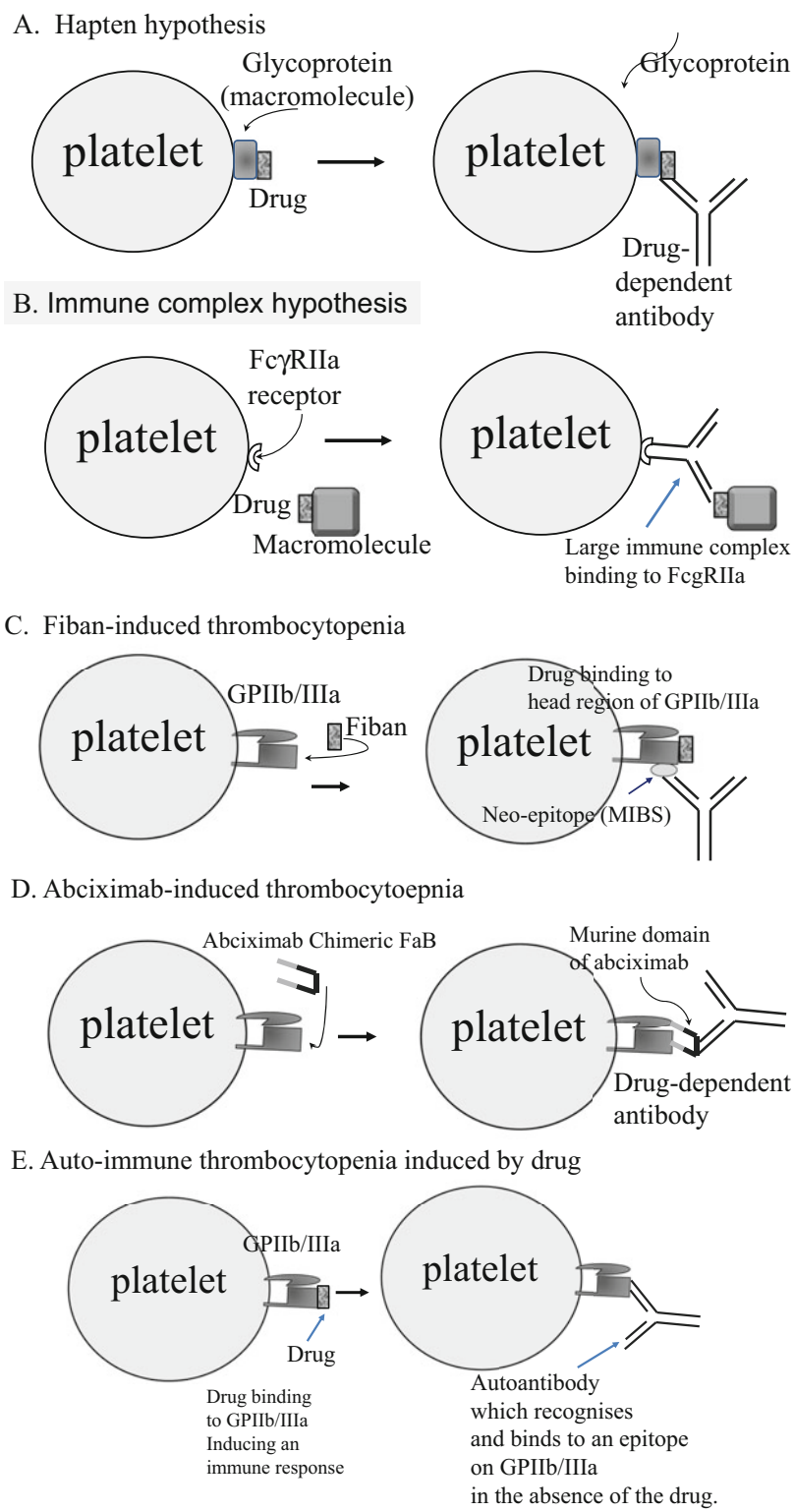

Fig. 2 Hypotheses of antibody-drug-glycoprotein interaction (continued). (**a**) *Hapten hypothesis:* Drug (so small to be immunogenic) binds to a macromolecule and induces an immune reaction resulting in the production of an antibody that binds strongly to the macromolecule only in the presence of the drug. (**b**) *Immune complex or* "*innocent-bystander*" *hypothesis*: As in the hapten hypothesis, the drug binds to a macromolecule and induces an immune response leading to the production of an antibody that the binds to the macromolecule-drug complex to form a large immune complex which then binds to platelet via its FcγRIIa receptor. (**c**) *Fiban-induced thrombocytopenia*: Fiban (drug) binds to the RGD site in the head region of platelet $\alpha_{IIb}\beta_3$ and induces a conformational change resulting in the emergence of a neoepitope (also known as MIBS) to which the drug-dependent antibody binds. (**d**) Abciximab-induced thrombocytopenia: Abciximab (a human-murine chimeric Fab) binds to the RGD recognition site at the head region of platelet $\alpha_{IIb}\beta_3$. The abciximab-dependent antibody has specificity for the murine domain of abciximab and it binds to this domain of the drug. (**e**) Drug-induced autoimmune thrombocytopenia. Anti-platelet autoimmune antibody binds to platelets in the absence of the drug

onset of thrombocytopenia is delayed till 5–8 days after drug administration (Jenkins et al. 1998).

The acute thrombocytopenia is believed to be due to natural occurring antibodies which have specificity for the murine sequences in the chimeric Fab (Curtis et al. 2002, 2004) (Fig. 2d). The delayed thrombocytopenia is mediated by an induced antibody that reacts with the platelet-bound drug which can persist for weeks (Curtis et al. 2004; Mascelli et al. 1998). Some of these antibodies may also cause platelet activation. However,

the antibody has also been found in healthy volunteers without previous drug exposure, but the antibody specificity is different as this antibody recognizes the papain cleavage site introduced during the manufacture of the drug (Curtis et al. 2002).

The thrombocytopenia caused by abciximab and other $\alpha_{IIb}\beta_3$ inhibitors can be severe and the potential bleeding risk is high, particularly in the clinical setting when patients also receive other anti-platelet agents (aspirin and clopidogrel) and frequently have undergone invasive revascularization procedures. However, the actual incidence of bleeding is surprisingly low. Nevertheless, thrombocytopenia in this cardiovascular setting is associated with unfavourable clinical outcomes including increased mortality rate, higher incidence of acute myocardial infarction and increased frequency of blood transfusions (Sinkovic and Majal 2015).

5. *Autoimmune thrombocytopenia induced by drugs*. Autoimmune haemolytic anaemia induced by drugs such as alpha methyldopa, procainamide and levodopa is well described (Petz 1993), but autoimmune thrombocytopenia caused by these and other drugs is less well characterized (Giner et al. 2003; Aster 2000; Landrum et al. 1994). Besides the three drugs, a number of other drugs have also been reported to induce autoimmune thrombocytopenia. These other drugs include (1) gold salts used previously to treat rheumatoid arthritis (Adachi et al. 1987; von dem Borne et al. 1986; Garner et al. 2002; Griem and Gleichmann 1995), (2) sulfamethoxazole (Aster 2000) and (3) monoclonal antibodies used to treat cancers such as rituximab (Ram et al. 2009), efalizumab (Warkentin and Kwon 2005) and trastuzumab (Drudi et al. 2010).

   In contrast to other DITPs, the platelet-specific antibodies produced react with platelets in the absence of the drug (Fig. 2e), similar to the autoantibodies in primary immune thrombocytopenia (ITP). In gold-induced thrombocytopenia, the antibodies were reported to have specificity for $\alpha_{IIb}\beta_3$ (von dem Borne et al. 1986), but one study found that antibodies in this condition are predominantly GPV specific (Garner et al. 2002). The mechanism whereby the drugs induce production of platelet-specific autoantibodies is still unclear although heavy metals have been shown to trigger autoimmunity in animal models (Griem and Gleichmann 1995). There is also a hypothesis that suggests that gold might interfere with macrophage processing of platelet membrane glycoproteins in such a way that "cryptic peptides" normally not seen by the immune system are generated and they trigger an immune response.
6. *Immune complex mechanism*. Before the 1980s, this mechanism, previously known as "innocent-bystander" mechanism, was thought to be the pathogenic process causing immune thrombocytopenia induced by quinine and most other drugs (Ackroyd 1953; Shuman 1958, 1964). Since the 1980s, however, there has been a large body of evidence against this mechanism being the cause of thrombocytopenia induced by quinine (Chong et al. 1983, 1991; Smith et al. 1987; Christie et al. 1985) and other drugs (Visentin et al. 1991; Gentilini et al. 1998). In the immune complex mechanism, the drug attached to a macromolecule (a plasma protein) and the antibody produced following drug sensitization reacts with the drug and macromolecule to form an immune complex which binds to platelets via their FcγRIIa receptors (Fig. 2b). Cross-linking platelet FcγRIIa receptors leads to platelet activation and adverse clinical consequences such as thrombosis (Chong and Isaacs 2009; Shantsila et al. 2009).

   The *immune complex mechanism* is applicable to thrombocytopenia induced by only a very small number of drugs, most notably heparin and similar drugs like pentosan polysulphate and protamine sulphate (Singla et al. 2013; Bakchoul et al. 2013; Goad et al. 1994; Schwartsmann et al. 1996; Pluda et al. 1993; Tardy-Poncet et al. 1994). The drug-dependent antibodies in protamine-induced thrombocytopenia have recently been characterized. Unlike the antibodies in HIT, the protamine-dependent antibodies appear to recognize a neoepitope on protamine (positively charged) after the drug has complexed with heparin or platelet surface glycosaminoglycan (both negatively charged); no plasma protein-like platelet factor 4 (PF4) is involved (Singla et al. 2013; Bakchoul et al. 2013). The immune complex formed induces platelet activation (e.g. serotonin release) via its binding to and cross-linking of platelet FcγRIIa receptors. This can be blocked by anti-FcγRIIa receptor monoclonal antibody, IV.3. Similarly, pentosan polysulphate (Goad et al. 1994), a negatively charged heparinoid, used in clinical trials for treatment of advanced malignancies such as human immunodeficiency virus-associated Kaposi's sarcoma has been reported to cause thrombocytopenia and thrombosis via the immune complex mechanism (Schwartsmann et al. 1996; Pluda et al. 1993; Tardy-Poncet et al. 1994).

HIT specifically will not be discussed in this chapter but will be covered in Greinacher et al. (2017).

Immune Processes that Increase in Platelet Clearance and Suppression of Platelet Production Resulting in Thrombocytopenia

1. *Increase in platelet clearance by drug-dependent antibodies and drug*

   It is widely believed that antibody-coated platelets are cleared rapidly by macrophages in the spleen and/or liver

by phagocytosis via Fc receptors. This notion probably arises from platelet kinetic studies in patients with ITP in the 1970s and 1980s which showed shortened platelet survival in ITP patients, increased platelet clearance and platelet turnover (Branehog et al. 1974; Harker 1970). The increased platelet turnover was believed to be a compensation for the increased platelet loss. However, there is no similar human data for DITP. We and Bougie et al. have provided data from murine DITP models (Bougie et al. 2010; Liang et al. 2010). Both groups showed rapid platelet clearance mediated by quinine-dependent antibodies in NOD-SCID mice. Human platelets injected immediately after administration of DITP serum/IgG and quinine were rapidly cleared from the circulation in these mice. The profound and rapid platelet clearance was seen uniformly with sera/IgG of seven patients with quinine-induced thrombocytopenia (Liang et al. 2010). This is consistent with the classical clinical picture of severe thrombocytopenia of rapid onset in patients with thrombocytopenia induced by quinine. In contrast, platelet clearance mediated by sera/IgG of primary immune thrombocytopenia (ITP) was more variable and gradual and less marked, in keeping with the heterogeneous nature of chronic ITP.

Platelet lysis by complement, T cells and radical oxygen species (ROS) and platelet clearance via hepatic Ashwell-Morrell receptors have been demonstrated in ITP (Najaoui et al. 2012; Olsson et al. 2003; Nardi et al. 2001, 2004; Li et al. 2015), but there is no evidence that these mechanisms also cause platelet destruction or clearance in DITP.

2. *Inhibition of platelet production by drug-dependent antibodies and drug*

Platelet kinetic/turnover study by Ballem et al. (1987) has shown decreased platelet production in a significant number of patients with chronic ITP, and this was attributed to megakaryocyte immuno-injury induced by anti-platelet antibodies (Piguet and Vesin 2002). These autoantibodies have specificity of GPIb-IX and/or $\alpha_{IIb}\beta_3$ which are present not only on platelets but also on megakaryocytes. It is not unexpected that these antibodies bind and inflict immuno-injury to megakaryocytes. Hoffman et al. (1985), Takahashi et al. (1999), McMillan et al. (2004) and Chang et al. (2003) demonstrated that ITP autoantibodies and monoclonal antibodies against platelet glycoproteins impaired proliferation and maturation of megakaryocytes in in vitro culture. ITP autoantibodies also blocked proplatelet formation and platelet release by cultured megakaryocytes (Li and Kuter 2001; Iraqi et al. 2015). However there are only limited number of studies on the effects of drug-dependent antibodies on megakaryocytes and platelet production. Greinacher et al. (2009) found that epifibatide-induced antibodies enhanced megakaryocyte apoptosis and impaired their proliferation and consequently prolonged the thrombocytopenia. We provided further evidence of immuno-injury to megakaryocytes by drug-dependent antibodies (Perdomo et al. 2013). We also demonstrated that the quinine-induced antibodies not only bound in the presence of quinine to megakaryocytes but they led to cell apoptosis, decreased cell vitality and increased cell death. Quinine-induced antibody binding also decreased significantly GPIX/GPIbα-bearing megakaryocytes (mature megakaryocytes) suggesting that antibodies impaired terminal megakaryocyte differentiation. Furthermore, when megakaryocytes were cultured in vitro with quinine-dependent antibody and quinine, proplatelet formation and platelet production were significantly inhibited (Perdomo et al. 2013).

Diagnosis of DITP

Clinical History of Drug Taking

A patient with an unexpected severe thrombocytopenia of sudden onset should prompt the clinician to consider the possibility of DITP. The patient should be asked about taking not only drugs that commonly cause thrombocytopenia but also herbal medicine, certain foods and tonics that may cause the same side effect. Quinine is present in some beverages such as tonic water and Schweppes Bitter Lemon (Korbitz and Eisner 1973; Brasic 2001). *Jui*, a Chinese herbal medicine, and food items such as tahini have also been reported to induce significant reduction in blood platelets (Arnold et al. 1998; Azuno et al. 1999; Ohmori et al. 2004).

Clinical Picture

The clinical picture varies with the inducing drug and the severity of the thrombocytopenia. For most DITP, the thrombocytopenia is severe and patients often present with extensive petechiae and bruises and oral blood blisters. Mucosal bleeding and occasionally intracranial haemorrhage may also occur. Fatal bleeding may happen but fortunately only infrequently. The onset is often abrupt, usually occurring 5–14 days after drug commencement, but early onset can occur if there is a previous drug exposure. In contrast, DITP caused by $\alpha_{IIb}\beta_3$ inhibitors (fibans and abciximab) presents with severe thrombocytopenia of sudden onset usually within 2–48 h of drug administration (Jubelirer et al. 1999; Curtis et al. 2002, 2004; Kilickiran et al. 2008). This often happens in patients with no previous exposure to the drug. However, in some patients the onset of

thrombocytopenia may be delayed until 5–8 days after abciximab administration (Jenkins et al. 1998).

For diagnosis of DITP, two sets of clinical data should be considered:

(a) Patient-specific data
(b) Information regarding the capacity of the drug to cause DITP

(a) *Patient-specific data*. The clinician should consider whether the temporal relationship of the drug commencement or the onset of thrombocytopenia is consistent with the drug causing DITP. The caring physician should also be aware of drug interactions that may cause DITP. A drug may increase in the plasma concentration of the metabolite of another drug which induces thrombocytopenia (Pandey and Yarlagadda 2012). This is particularly important in patients with impaired renal or liver functions. An example is dexamethasone and phenytoin (see above, Sect. "Drugs that Cause DITP"). Other drugs and co-morbid conditions that may cause thrombocytopenia should be excluded. Exclusion of other causes, particularly other concomitant drugs, may be difficult. Each suspected drug may be stopped one at a time, but this may take too long. It may be more practical to stop all suspected drugs until the offending drug is identified by a laboratory assay (see below) or a re-challenge with the suspected drug is carefully undertaken. Drug re-challenge is seldom carried out nowadays as it incurs too high a risk to the patient. Except for those drugs that cause autoimmune thrombocytopenia such as gold salts, procainamide and rituximab (von dem Borne et al. 1986; Ram et al. 2009; Singla et al. 2013), the return of the platelet to normal levels or baseline after cessation of the suspected drug provides direct confirmation of the diagnosis of DITP. Clinical criteria for diagnosis of DITP listed in Table 1 may be helpful.

(b) *Capacity of the suspected drug to cause DITP*. The potential of a suspected drug to cause thrombocytopenia should be sought from previous published studies (George et al. 1998) and certain websites, in particular the strength of evidence and the frequency of the drug causing DITP. A helpful website is http://www.ouhsc.edu/platelets. This website provides up-to-date information regarding all case reports of DITP and objective assessment of the level of evidence for a particular drug to cause DITP.

Table 1 General mechanisms of drug-induced immune thrombocytopenia

Major mechanisms	Hypotheses
Increase in platelet clearance	• Increased platelet clearance by *splenic and *hepatic macrophages • *Increased platelet clearance via hepatic Ashwell-Morrell receptors • *Platelet lysis by complements in circulation • #Platelet lysis by free oxygen radicals in circulation
Suppression of platelet production	• Suppression by megakaryocyte differentiation • Increase in megakaryocyte apoptosis • Inhibition of proplatelet production

Some evidence in *ITP or #thrombocytopenia associated with HIV but no direct evidence yet in DITP

Laboratory Diagnosis

The clinical diagnosis of DITP should be confirmed whenever possible by the detection of specific drug-dependent antibody using a laboratory assay. Before testing of drug-dependent antibodies, it is important to exclude the possibility of pseudothrombocytopenia (Barton and Stack 2009). Pseudothrombocytopenia is an in vitro artefact due to platelet clumping, leading to falsely low automated platelet counts with blood samples anticoagulated with ethylenediaminetetraacetic acid (EDTA). The incidence of pseudothrombocytopenia is increased from a baseline of 0.1–1.1 % in patients receiving abciximab. If pseudothrombocytopenia is confirmed by observation of platelet clumping on blood smear examination, the platelet count should be repeated by retaking of blood sample using a different anticoagulant such as citrate. In the absence of EDTA, the EDTA-dependent anti-platelet antibody does not induce platelet clumping.

There are two approaches to detect anti-platelet antibodies: (1) direct test and (2) indirect test (Chong and Keng 2000; Chong 2008; Heikal and Smock 2013).

1. *Direct test* detects antibody (IgG) on or associated with the patients' platelets. For this approach, the patient platelets are used and a fluorescence-labelled or enzyme-linked anti-human IgG or IgG/IgA/IgM secondary antibody is then used to detect the antibody on patient platelets often with flow cytometry or enzyme-linked immunosorbent assay (ELISA). Isotope-based assay such as the ^{51}Cr platelet lysis test is rarely used nowadays (Cimo et al. 1977) because of its lack of sensitivity and also the hazard of radioactivity it imposes.

   Normal platelets are used as a negative control. This method is suitable for detecting autoantibodies in primary ITP or DITP caused by drugs which induced autoimmune thrombocytopenia such as gold salt, methyldopa and rituximab (see Sect. "How Does the Drug-Dependent Antibody Bind to Platelets in the

Presence of the Drug?"). It has disadvantages of (a) being unable to determine the drug dependency of drug-related anti-platelet antibodies and thus unable to identify the offending drug and (b) the difficulty of obtaining enough patient platelets for testing if the thrombocytopenia is severe. This test is not usually used in DITP unless the patient has drug-induced autoimmune thrombocytopenia. One should however be aware that in some patients with DITP, a weak autoantibody is sometimes present in addition to the stronger drug-dependent antibody (Lerner et al. 1985; Bougie et al. 2006).

2. *Indirect test* detects antibody in the patient's serum. In the indirect test, patient serum is incubated with normal group O platelets in the presence or absence of the suspected drug (Heikal and Smock 2013). The antibody bound drug dependently to normal platelets is then detected using a fluorescence-labelled or enzyme-linked anti-human IgG or IgG/IgA/IgM secondary antibody as in the direct test. The indirect test is usually used for DITP. The blood sample should be collected after drug withdrawal when the suspect drug is no longer present in the patient serum. The assay can be repeated with each suspected drug to identify the offending drug. This is often dubbed as the "in vitro drug re-challenge". In some instances, the inducing agent is the drug metabolite, in which case the drug metabolite should be used instead of the parent drug. Positive result of the sample with normal platelets and patient serum in the absence of the suspected drug suggests that the antibody detected is an autoantibody. Another negative control used is normal platelets, plus normal serum and suspected drug; this is to exclude non-specific reactions such as drug autoflourescence.

The common methods commonly used to detect the drug-dependent antibody either by the direct or indirect test are:

1. Flow cytometry
2. ELISA
3. Monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay

The *MAIPA assay* has the advantage of being able to identify the platelet glycoprotein which is the target of the drug-dependent antibody. Each assay can be designed to detect IgG, IgM or IgA antibody separately or altogether.

Unlike autoantibody assays for diagnosis of ITP, drug-dependent antibody assays are often very helpful in the diagnosis of DITP, particularly for thrombocytopenia caused by quinine, quinidine, rifampicin, vancomycin, fiban and abciximab (Chong et al. 1983, 1991; Burgess et al. 2000; Asvadi et al. 2001; Bougie et al. 2002; Curtis et al. 2004). In our experience, these assays have high sensitivity and specificity. Flow cytometry is available in most clinical laboratories although MAIPA may be technically demanding and its availability may be somewhat restricted.

Management

The immediate step in the management of DITP is the cessation of the suspected inducing drug or in some cases the herbal medicine, tonic or food item. It must be noted that sometimes two drugs may cause DITP at the same time and both drugs should be withdrawn (Anand and Chauhan 2011). After drug withdrawal, the drug-dependent antibody does not bind to platelets or megakaryocytes even though the antibody may persist for several months thereafter. High-dose corticosteroid such as prednisone (1–2 mg/kg) and IVIg (2 g/kg over 2–5 days) is often administered when the thrombocytopenia is severe and risk of bleeding high (Chong 1991; Aster et al. 2009; George et al. 2006). The thrombocytopenia invariably resolves in 1–2 weeks or longer if the drug has a prolonged plasma half-life or there is impaired renal function. Therefore it is difficult to know if these treatments are efficacious. However, Liang et al. (2010) provided evidence in a murine model of DITP that IVIg was partially effective in preventing rapid platelet clearance by drug-dependent antibodies and improving platelet survival. The mechanism whereby IVIg exerts its effect is unclear in DITP; it may induce Fc receptor blockage or may impair antigen-presenting cell processing (Chong and Chong 2010).

It should be noted that if serious bleeding such as intracranial haemorrhage occurs when the platelet count is markedly low within the first few days after drug withdrawal, there is no effective treatment that will increase the platelet count. Nevertheless, platelet transfusion is usually given, preferably after Fc receptor blockage by IVIg infusion, but the transfused platelets are often rapidly cleared. Red cell transfusion should also be administered if the Hb level is 80 g/L or lower. Anti-fibrinolytic therapy such as tranexamic acid is helpful. Dialysis or plasma exchange to remove the offending drug may be needed in the case of a drug with very long plasma half-life or in patients with severely impaired renal function. In patients with DITP, in contrast to ITP, corticosteroid should be abruptly stopped when the thrombocytopenia has resolved.

In patients with DITP due to $\alpha_{IIb}\beta_3$ inhibitors, there may be other management issues. These drugs are frequently administered to patients with high-risk acute coronary syndrome or with incipient/overt heart failure. Thrombocytopenia in these patients is associated with increased mortality

Table 2 Hypotheses of platelet-antibody-drug interactions in DITP

Hypotheses	Descriptions
Hapten mechanism	The drug is too small to be immunogenic. It binds covalently to an autologous protein and elicits an immune response. The antibody produced recognizes the specific protein only when the hapten is attached. A possible example is penicillin-induced thrombocytopenia (Fig. 2a)
Quinine-type DITP	1. *Hybrid paratope* mechanism. The drug binds to the CDR domain of a naturally occurring antibody that has a weak affinity to the platelet GPIIbβ. It induces a conformational change in the CDR of the antibody transforming the antibody to one that binds to platelet GPIIbβ with high affinity and specificity (Fig. 1a) 2. *Neoepitope* mechanism. The drug binds weakly and non-covalently to a hyperflexible domain of a platelet receptor (GPIX or GPIIIa), inducing emergence a cryptic or new epitope never before seen by the immune system and eliciting an immune response. The antibody produced recognizes the neoepitope and binds with high affinity and specificity (Fig. 1b)
Fiban-induced DITP	Fiban (drug) binds platelet $\alpha_{IIb}\beta_3$ near the RGD binding site, inducing a conformational change and formation of a neoepitope (MIBS) (see text) to which antibody binds (Fig. 2c)
Abciximab-induced DITP	The patient's naturally occurring antibody recognizes and binds to a mouse sequence of abciximab chimeric Fab which is attached to platelets (Fig. 2d)
Autoimmune thrombocytopenia induced by drugs	Drug ingestion affects the immune system in such a way that autoimmunity results. Anti-platelet autoantibody produced reacts with platelets in the absence of the drug (Fig. 2e)
Immune complex mechanism	The antibody binds to a plasma protein-drug complex and the immune complex binds to the platelet via its FcγRIIIa receptor, and the platelet is destroyed/activated as an innocent bystander (Fig. 2b)

and other complications. Red cell transfusion should be considered at Hb levels lower than 80 g/L to improve tissue oxygen delivery, particularly in the setting of coronary ischaemia. In addition to the management described above, withdrawal of anti-platelet agents and anticoagulants, if clinically appropriate, should be considered in the event of serious bleeding (Tables 2 and 3).

Future Directions

Quinine-induced thrombocytopenia is the prototype of DITP caused by most drugs. Hybrid paratope hypothesis is the proposed mechanism supported by strong experimental evidence. Yet, there are still unanswered questions regarding this hypothesis such as:

(1) How does quinine sensitization occur? (2) Is the immunogen a complex resulting from weak, non-covalent binding of quinine to GPIIb? (3) Does the hybrid paratope hypothesis also apply to quinine-dependent antibodies that have specificity for GPIbα, GPIX and GPIIIa?

Answers to these questions await future studies. Further studies are also required to confirm the hypothesis of fortuitous binding of quinine to the B cell receptor as the initiating event for the production of quinine-induced anti-platelet antibodies.

There is an unmet clinical need for an effective treatment when serious bleeding occurs before platelet recovery in patients with DITP, particularly when the offending drug has a prolonged plasma half-life or drug clearance is delayed due to impaired renal or liver functions. Future research may lead to development of efficacious treatments that will rapidly increase circulating platelets during the thrombocytopenic phase of DITP.

Table 3 Drugs with strong evidence that they cause drug-induced immune thrombocytopenia

References	Reese et al. (2010))	Arnold et al. (2013)
Implicated drugs	Abciximab	Abciximab
	Acetaminophen	–
	Amiodarone	–
	Ampicillin	–
	Carbamazepine	Carbamazepine
	–	Ceftriaxone
	Eptifibatide	Eptifibatide
	Ethambutol	–
	Haloperidol	–
	–	Heparin
	Ibuprofen	Ibuprofen
	Irinotecan	–
	–	Mirtazapine
	Naproxen	–
	Oxaliplatin	Oxaliplatin
	–	Penicillin
	Phenytoin	–
	Piperacillin	–
	Quinidine	Quinidine
	Quinine	Quinine
	Ranitidine	–
	–	–
	Simvastatin	–
	Sulfisoxazole	Rifampicin
	–	Surami
	Tirofiban	Tirofiban
	Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole
	Valproic acid	–
	Vancomycin	Vancomycin

The two lists of drugs overlap with a number of drugs appearing on both lists, but some drugs appear in one but not in the other

Conclusion

In recent years, there has been considerable new knowledge of DITP pathogenesis, particularly thrombocytopenia caused by quinine (the prototype drug) and $\alpha_{IIb}\beta_3$ inhibitors (fibans and abciximab). Recent research has shown that the drug-dependent antibody binds not only to platelets inducing their rapid clearance but also to megakaryocytes causing apoptosis, inhibiting their maturation and platelet production. The binding sites of human drug-dependent antibodies, particularly quinine-dependent antibodies, have been fine mapped to a hyperflexible region in the membrane-proximal domain of GPIX (R110–Q115) and PSI domain of GPIIIa (A50–D66). However, the most exciting recent advance in DITP research is the finding of structural changes in the CDRs of the murine monoclonal quinine-dependent antibodies induced by quinine binding converting them to the antibodies that bind with high affinity and specificity to their target platelet glycoprotein (GPIIb). This finding leads to the unconventional hybrid paratope hypothesis. The challenge in future research is to reconcile this finding to previous observations in DITP.

Diagnosis of DITP is usually made clinically based on clinical diagnostic criteria. Whenever possible, the clinical diagnosis of DITP should be confirmed by detection of the drug-dependent antibodies. The crucial immediate step in DITP treatment is prompt cessation of the suspected drug (s) and thrombocytopenia invariably resolves within 1–2 weeks. Even though their efficacy is still unclear, high-dose prednisone and IVIg are usually administered during the period of severe thrombocytopenia because of the high risk of potentially catastrophic bleeding. Glucocorticosteroid is stopped abruptly once thrombocytopenia resolves.

Take-Home Message Boxes

Diagnosis of Drug-Induced Immune Thrombocytopenia (DITP)

- Clinical diagnosis:
  - Patient-specific data
    History of drug taking consistent with diagnosis of DITP
    History that satisfies one or more clinical diagnostic criteria (Table 4)
  - Consider the capacity of the drug to cause DITP.
- Laboratory diagnosis:
  - Detection of drug-dependent antibody or autoantibody

Table 4 Clinical diagnostic criteria

Criteria	Description
1	Suspected drug is commenced 5–14 days before the onset of thrombocytopenia. After suspected drug withdrawal, platelet count returns to normal or baseline levels
2	Suspected drug is the only drug administered before the onset of thrombocytopenia or other drugs are continued/reintroduced after withdrawal of suspected drug. Continuation/reintroduction of other drugs does not hinder platelet recovery after cessation of the suspected drug
3	Other causes of thrombocytopenia are excluded clinically
4	Re-challenge with the suspected drug results in prompt recurrence of thrombocytopenia or the offending drug is identified by detection of the specific drug-dependent antibody by a laboratory assay
	Level of evidence Definite: Criteria 1, 2, 3 and 4 met Probable: Criteria 1, 2 and 3 met Possible: Criteria 1 met Unlikely. Criteria 1 not met

Management of Drug-Induced Immune Thrombocytopenia (DITP)

- Cease the offending drug.
- If thrombocytopenia is severe and bleed risk is high/bleeding has occurred, commence high-dose corticosteroid therapy and/or IVIg.
- If bleeding is severe,
  - Give platelet transfusion preferably after IVIg has provided Fc receptor blockade.
  - Start anti-fibrinolyitc agent such as tranexamic acid.
- Give red cell transfusion if Hb drops below 80 g/L.

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Heparin-Induced Thrombocytopenia

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Abstract

Heparin-induced thrombocytopenia (HIT) is an adverse prothrombotic disorder caused by an immune response to complexes of platelet factor 4 and polyanions. The clinical relevance of HIT results from the wide use of heparin, which is the reason that the absolute number of patients affected by HIT is high. HIT is currently the most frequent immune-mediated adverse drug reaction affecting blood cells.

This chapter will address the clinical presentation of HIT and summarize recent findings on its pathogenesis including the concept that HIT is likely a misdirected bacterial host-defense mechanism. Description of laboratory tests for HIT and an outline on current recommendations for management of affected patients provide information relevant for daily clinical practice.

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Introduction

Thrombocytopenia is relatively common in hospitalized patients, especially in the critically ill. Although a falsely low platelet count can be caused by laboratory artifact ("pseudothrombocytopenia"), true thrombocytopenia has multiple potential explanations that can be classified in five general ways (Table 1). Timing of the onset of thrombocytopenia and its severity differ considerably among different causes (Table 2). Most immune-mediated thrombocytopenias are associated with an increased risk for bleeding, while thrombotic thrombocytopenic purpura and heparin-induced thrombocytopenia (HIT) increase the risk for thrombotic complications (although patients with TTP often present with bruising and mucosal bleeding). Table 3 summarizes the main features of immune thrombocytopenias.

Nonimmune Heparin-Associated Thrombocytopenia

Heparin binds to platelets, resulting in mild platelet activation (Salzman et al. 1980; Chong and Ismail 1989) and an enhanced aggregation response to several agonists (Chong

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Table 1 Six general explanations for thrombocytopenia, including pseudothrombocytopenia

Explanation	Comment	Examples
Pseudothrombocytopenia	Antibody-mediated platelet clumping that occurs ex vivo, i.e., spurious thrombocytopenia	EDTA-induced pseudothrombocytopenia; platelet satellitism (platelet binding to leukocytes)
Hemodilution	Abrupt platelet count fall due to administration of fluids or blood products	Early postoperative thrombocytopenia
Hypersplenism	Splenomegaly may require imaging studies to detect; concomitant leukopenia and (to lesser extent) anemia are usually present	Cirrhosis-associated portal hypertension
Decreased platelet production	Usually there is concomitant leukopenia and anemia (a notable exception is certain types of hereditary thrombocytopenia)	Myelodysplasia; aplastic anemia; marrow infiltration by metastatic cancer; alcohol-induced thrombocytopenia
Platelet consumption	Accelerated platelet clearance due to pathologically increased platelet activation or other poorly defined mechanisms of increased platelet clearance	Disseminated intravascular coagulation; septicemia; multiorgan system failure; hemophagocytic syndrome
Platelet destruction	Accelerated platelet clearance due to pathological mechanisms that target platelets, especially due to platelet-reactive antibodies	Autoimmune thrombocytopenic purpura; HIT; drug-induced immune thrombocytopenia; TTP/HUS

EDTA ethylenediaminetetraacetic acid, *HIT* heparin-induced thrombocytopenia, *TTP/HUS* thrombotic thrombocytopenic purpura/hemolytic uremic syndrome

and Castaldi 1986; Holmer et al. 1980). This effect is pronounced in patients with infection and peripheral vascular disease (Mikhailidis et al. 1985; Burgess and Chong 1997). Unfractionated heparin (UFH), low-molecular-weight heparin (LMWH), and fondaparinux induce outside-in signaling via the glycoprotein (GP) IIb-IIIa complex (Gao et al. 2011), which potentiates the effect of low-dose adenosine diphosphate (Gao et al. 2011). Whereas UFH and fondaparinux had similar proactivating effects in one study (Gao et al. 2011), another observed much less pronounced effects of LMWH compared to UFH (Barradas et al. 1987).

In vivo these proaggregatory effects can explain a moderate decrease in platelet count after therapeutic-dose UFH, less with LMWH (Horne III 2007), most likely due to mild platelet aggregation in vivo, with platelet aggregates cleared by the reticuloendothelial system (Balduini et al. 1993; Blauhut et al. 1985). This phenomenon might explain why an overall lower frequency of thrombocytopenia was seen in critically ill patients who received LMWH versus UFH in a randomized trial (Williamson et al. 2013). However, the frequency of nonimmune heparin-associated thrombocytopenia is difficult to discern, as there is no diagnostic test and hospitalized patients usually have alternative explanations for an early platelet count fall.

Immune HIT

Clinical Features

Thrombocytopenia

Typical- vs. Rapid-Onset HIT The platelet count usually falls beginning 5–10 days after the start of an immunizing exposure to heparin, most often intra/perioperative heparin (Fig. 1) (Warkentin and Greinacher 2003). The platelet count then declines substantially (usually >50 %) over the next 2–3 days. This pattern ("typical-onset" HIT) is seen in approximately 60–70 % of patients (Warkentin and Kelton 2001b). In 25–30 % of cases, onset of thrombocytopenia occurs within 24 h of starting heparin ("rapid-onset HIT") (Warkentin and Kelton 2001b); these patients have been previously exposed to heparin within the recent past (usually within the last month but sometimes up to 3 months). This profile is explained by heparin being restarted in a patient who already has circulating HIT antibodies, accounting for the abrupt platelet count fall. The strong association with recent prior heparin exposure reflects the striking transience of HIT antibodies (Warkentin and Kelton 2001b).

Delayed-Onset HIT Sometimes, thrombocytopenia begins several days after stopping the immunizing course of heparin or worsens in spite of stopping heparin ("delayed-onset HIT" or "autoimmune-like" HIT) (Warkentin and Kelton 2001a; Rice et al. 2002; Greinacher 2014). These patients have high-titer HIT antibodies that are somewhat heparin-"independent", i.e., the patient serum activates platelets strongly in vitro without requiring pharmacologic heparin.

Persisting HIT Rarely, thrombocytopenia persists for at least 1 month after stopping heparin (~1 % of patients) (Kopolovic and Warkentin 2014); in contrast, instances of platelet count recovery in spite of continued heparin have also been reported (Warkentin et al. 2013). These clinical extremes reflect differences in persistence of HIT antibodies with and without heparin-independent platelet-activating properties. In general, the median time to platelet count

Table 2 Thrombocytopenia (TP) in in-hospital patients in relation to the time course of platelet count

	Thrombocytopenia at admission	Normal platelet count at admission with initial platelet count fall and persistent thrombocytopenia for more than 4 days	New *rapid* platelet count fall after platelet recovery from on day 4 with no new major intervention	New *slow* platelet count fall after platelet recovery after day 4
Laboratory artifacts	– Pseudothrombocytopenia (preanalytical clot in blood tube) (⇔)		– Pseudothrombocytopenia (especially with $\alpha_{IIb}\beta_3$ inhibitor treatment) (preanalytical clot in blood tube) (⇔)	
Disturbed distribution	– Hemodilution (⇔⇧) – Hypersplenism (⇔⇧) – Chronic liver disease (⇔⇧)	– Circulatory shock (⇧)		– Circulatory shock (⇧)
Impaired platelet production	– Bone marrow toxicity (⇧–⇧⇧) Drugs, toxins, alcohol (⇧) – Myelodysplastic syndrome (⇧⇧) – Acute leukemia (⇧) – Viral infections (⇧) (HIV, HCV) – Chronic liver disease (⇧) – Hereditary (macro-) thrombocytopenia (⇧)	– Acute liver failure (⇧⇧) – Evolving bone marrow failure (⇧⇧)	– Acute liver failure (⇧⇧)	– Bone marrow toxicity (⇧⇧) Drugs Bacterial toxins – Liver failure (⇧⇧)
Enhanced consumption (nonimmune)	– Platelet loss after surgery or trauma (⇧) – Sepsis and DIC (⇧) – Severe thrombosis/ pulmonary embolism (⇔⇩) – Diabetic ketoacidosis – HELLP syndrome (⇔⇩)	– Ongoing bleeding (⇧⇧) – Sepsis and DIC (⇧⇩) – Multiorgan failure (⇧⇧) – Extracorporeal circuit (hemodialysis, continuous, hemofiltration, extracorporal oxygenation) (⇧⇧) – Hemophagocytosis syndrome – Postsurgery TTP (⇔⇩) – Bacterial contaminated blood transfusion (⇔)	– New bleeding (⇧) – Sepsis and DIC (⇧⇩) – Pulmonary embolism (⇔⇩)	– Chronic bleeding (⇧⇧) – Sepsis and DIC (⇧⇩)
Enhanced consumption (immune)	– ITP (⇔⇧) – Drug-dependent TP (⇧⇧) – $\alpha_{IIb}\beta_3$-inhibitor induced TP (⇧) – Thrombotic thrombocytopenic purpura (⇔⇩) – HIT if heparin has been given during the last 10 days (⇔⇩) – Antiphospholipid syndrome (⇔⇩) – Viral infections (HIV, HCV) (⇧)	– Postsurgery TTP (⇔⇩) – Evolving catastrophic anti-phospholipid syndrome (⇔⇩)	– HIT (⇔⇩) – $\alpha_{IIb}\beta_3$-inhibitor induced TP (⇧) – Drug-dependent TP (⇧⇧) – TTP (⇧ and ⇩) – Antiphospholipid syndrome (⇔⇩) – Passive transfusion of platelet allo-antibodies (⇧⇧) – Posttransfusion purpura (⇧⇧)	

Bleeding risk: ⇧⇧—strongly increased, ⇧—moderately increased, ⇔—not increased, ⇩—prothrombotic risk)

recovery ($>150 \times 10^9$/L) is 4 days, with 90 % showing platelet count recovery within 1 week (Warkentin 2013a; Greinacher et al. 2009; Warkentin et al. 2013).

Spontaneous HIT Rarely, a disorder occurs that clinically and serologically resembles HIT, except that there is no proximate heparin exposure; patients often have preceding infection or knee replacement surgery, suggesting that bacterial or knee cartilage-derived polyanions or free DNA/RNA rarely can trigger a strong HIT-mimicking immune response (Warkentin et al. 2008a, 2014).

Table 3 Immune thrombocytopenias

	ITP	PTP	Drug-dependent TP	GP IIb/IIIa inhibitor-TP	HIT	TTP
Platelet count	Variable <20,000	<10,000	<10,000	<10,000	40–80,000	10–30,000
Bleeding symptoms	(+) to +++	++++	++++	(+)	−−−	+−
Onset	Chronic	Day 7–14 after transfusion	Day 7–14 after start of drug (day 1 in case of reexposure)	Day 1 of $\alpha_{IIb}\beta_3$ treatment (delayed onset)	Day 5–14	Acute Deteriorating
Thrombosis	−− (+)	−−	−−	−+ Depends on treatment	++++	++

ITP autoimmune thrombocytopenia, *PTP* posttransfusion purpura, *TP* thrombocytopenia, *GP* glycoprotein, *HIT* heparin-induced thrombocytopenia, *TTP* thrombotic thrombocytopenic purpura

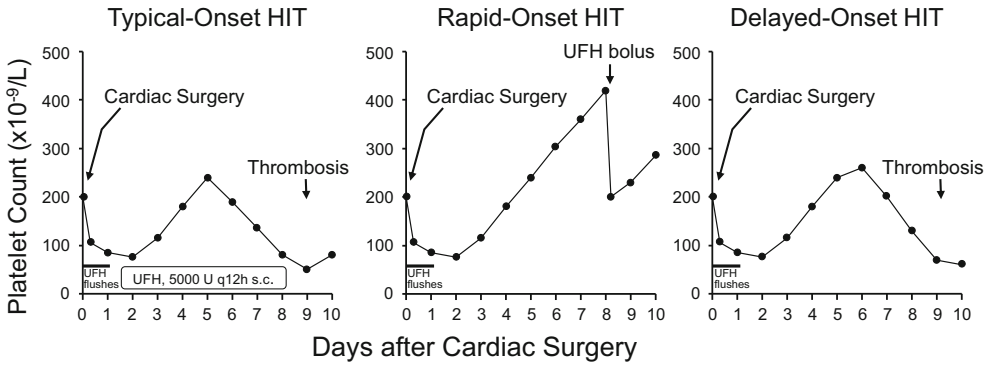

Fig. 1 Timing of onset of HIT. Temporal profiles of onset of HIT. Cardiac surgery patients are shown; thus, day of surgery = day 0, reflecting (immunizing) intraoperative exposure to UFH (unfractionated heparin). Modified from Warkentin TE and Greinacher A. Heparin-induced thrombocytopenia and cardiac surgery. *Ann Thorac Surg* 2003; 76 (6): 2121–2131 (Warkentin and Greinacher 2003)

Severity of Thrombocytopenia The thrombocytopenia of HIT is usually moderately severe (median platelet count nadir, ~60 × 10^9/L; Fig. 2) (Warkentin 2006), only rarely falling to <10 × 10^9/L; this contrasts from classic drug-induced immune thrombocytopenia (e.g., quinine, vancomycin), where the platelet count nadir is usually <20 × 10^9/L (Warkentin 2003, 2007). Sometimes, the platelet count falls by >50 %, but because of postoperative thrombocytosis, the platelet count nadir remains >150 × 10^9/L (Warkentin 2004).

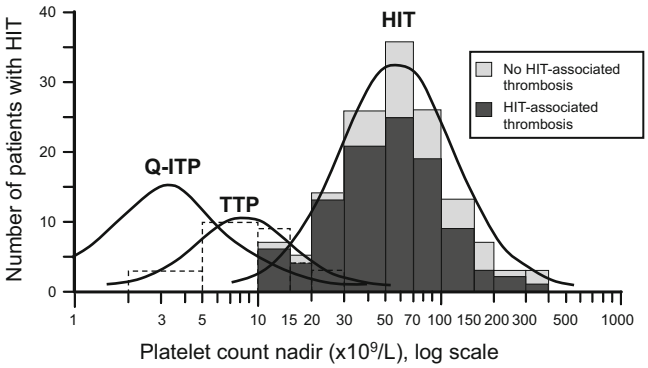

Fig. 2 Distribution of platelet count values in different disorders. HIT (with or without associated thrombosis), quinine-induced immune thrombocytopenia (Q-ITP), and thrombotic thrombocytopenic purpura (TTP) with absent ADAMTS-13 activity (dITP, TTP). From: Warkentin TE. Think of HIT. *Hematology Am Soc Hematol Educ Program* 2006; 408–414 (Warkentin 2006)

Thrombotic Complications

Thrombotic complications occur in 50–70 % of patients with serologically confirmed HIT (Warkentin 2003; Warkentin et al. 2013). Thrombosis can be venous, arterial, and/or microcirculatory. The overall ratio of venous/arterial thrombosis is ~4:1. Bleeding is uncommon.

Venous Thrombosis

Lower-limb deep-vein thrombosis (DVT), sometimes bilateral, is the most common HIT-associated thrombotic complication; pulmonary embolus is also relatively common (Warkentin 2013a). When microvascular thrombosis occurs in a limb with DVT, *phlegmasia cerulea dolens* (severe venous limb ischemia) can progress to *venous limb gangrene* (the most common cause of limb amputation in HIT) (Warkentin et al. 1997; Warkentin 2015c). Progression of DVT to venous limb gangrene typically occurs after stopping heparin and is strongly associated with vitamin K antagonist (VKA) therapy; patients have a characteristic "supratherapeutic" INR (>4) (Warkentin et al. 1997; Srinivasan et al. 2004). The pathogenesis is VKA-induced protein C depletion, with continuing HIT-associated thrombin generation, leading to microthrombosis.

Venous thrombosis can also occur in upper limb veins (at central venous catheter sites) (Hong et al. 2003), cerebral veins/dural sinuses (Meyer-Lindenberg et al. 1997), mesenteric veins, and adrenal veins (Arthur et al. 1985; Warkentin et al. 2015a); this last complication results in adrenal hemorrhage which—when bilateral—can cause life-threatening adrenal failure.

Arterial Thrombosis

Arterial thrombosis in HIT usually involves large arteries such as the distal aorta and lower-limb arteries (acute limb ischemia with absent pulses) (Rhodes et al. 1973); stroke, myocardial infarction, upper limb ischemia, and bowel infarction are other frequent events (Warkentin 2013a).

Microthrombosis

Overt (decompensated) DIC occurs in 10–20 % of patients with HIT (Warkentin 2013a) usually with severe thrombocytopenia and coagulopathy (e.g., elevated INR). Fibrin D-dimer levels are typically very high, but overt hypofibrinogenemia is uncommon, probably because fibrinogen levels are expected to be elevated in the postoperative patient who gets HIT. DIC is a risk factor for microthrombosis and limb ischemic necrosis, partly because of inappropriate direct thrombin inhibitor dose reduction or interruption because of falsely high APTT values (Warkentin 2015c) ("APTT confounding").

Other HIT-Associated Thrombotic Complications

Thrombosis of intravascular prostheses and hemodialysis circuit clotting can indicate HIT (Warkentin 2015a) as do necrotizing skin lesions at UFH or LMWH injection sites (Warkentin 1996a). Rarely, HIT is associated with skin necrosis in the absence of subcutaneous injections or VKA therapy (Tassava and Warkentin 2015).

Acute Systemic (Anaphylactoid) Reactions

Acute systemic (or anaphylactoid) reactions present as fever/chills, tachycardia, flushing, blood pressure changes, headache, chest pain, and dyspnea, beginning 5–30 min after an IV bolus of heparin or 30–120 min post-subcutaneous LMWH injection. Patients have circulating HIT antibodies, and there is a concomitant abrupt platelet count fall (Warkentin and Greinacher 2009) postinjection. Transient global amnesia has also been reported (Warkentin et al. 1994b).

Pathogenesis

HIT is mediated by platelet-activating IgG antibodies induced by polyanions, most often heparin (Fig. 3). Neoepitopes are formed on the cationic chemokine, platelet factor 4 (PF4; CXCL4), when PF4 forms complexes with polyanions (Amiral et al. 1992). PF4 is a positively charged tetrameric 35 kDa protein stored in platelet α-granules and secreted during platelet activation. PF4 binds to endothelial cell heparan sulfate, from which it detaches with heparin infusion.

The HIT Antigen and Antibodies

PF4 PF4 binds to platelets, e.g., via chondroitin sulfate. PF4 binding to heparin is greatest at an optimal stoichiometric ratio (PF4/heparin ~2:1 (Greinacher et al. 2008)). Binding of heparin to PF4 neutralizes its strong positive charge (Rauova et al. 2006), allowing two PF4 tetramers to come into close proximity (Greinacher et al. 2006). Besides the stoichiometric considerations, polyanion composition, chain length, and degree of sulfation also affect binding to PF4 (Horne and Chao 1990; Greinacher et al. 1995). In most clinical settings, plasma UFH and especially LMWH concentrations are in excess to the optimal concentration (Greinacher et al. 2008); this changes when a large number of activated platelets release PF4, helping explain why anti-PF4/heparin antibodies are more frequently produced in patients undergoing major surgery (Warkentin et al. 1995; Visentin et al. 1996) compared to medical patients.

The neoepitopes to which anti-PF4/heparin antibodies bind are only exposed on PF4 in its tetrameric form (Sachais et al. 2012), probably requiring at least three PF4 monomers (Block et al. 2014). A crucial requirement for a PF4/polyanion complex to expose neoepitopes is formation of at least three bonds between PF4 and polyanion. This induces a structural change in PF4, increasing to >30 % the content of antiparallel β-sheets (Brandt et al. 2014). Further, the reaction must release enough energy to support a further conformational change in PF4. Only heparin molecules with chain length ≥10 monosaccharides fulfill these requirements. Short heparins bind to only one PF4 tetramer, whereas long heparins bind to at least two PF4 tetramers. When two PF4 tetramers are brought closely together, the individual clouds of positive charge around each single PF4 tetramer fuse into one large cloud surrounding all of the PF4 molecules within the cluster (Nguyen et al. 2015). This charge redistribution provides the additional energy needed to expose the neoantigens. Formation of ultralarge PF4-polyanion complexes occurs especially with UFH (vs. LMWH), and does not occur with the

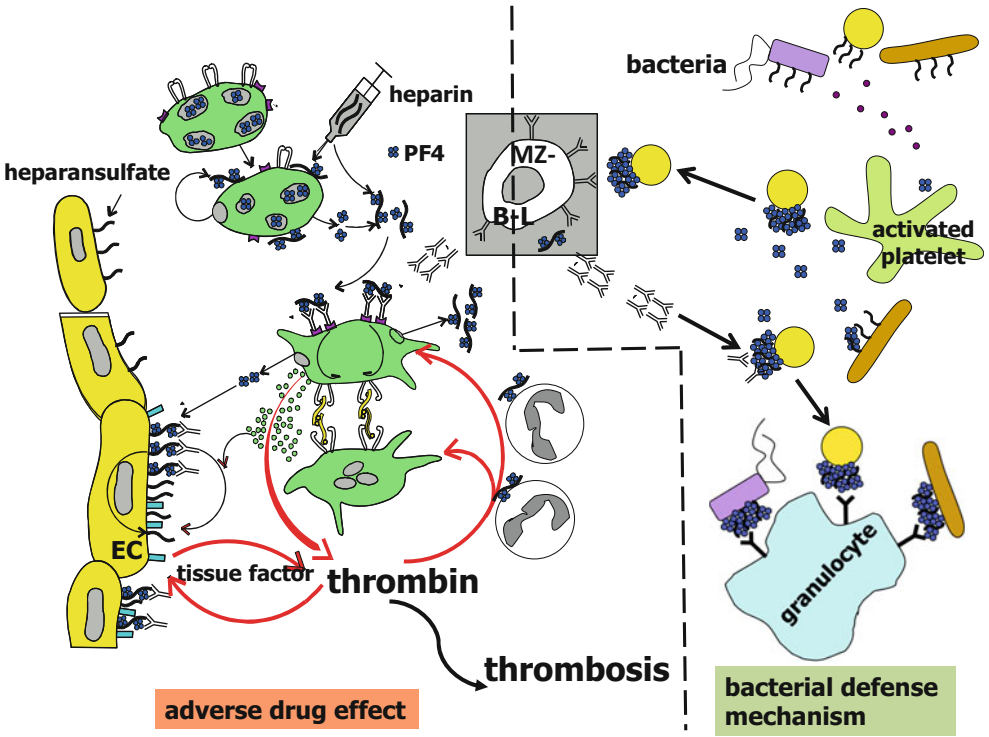

Fig. 3 Pathogenesis of heparin-induced thrombocytopenia: *left side*, in HIT the combination of anticoagulant treatment and thrombocytopenia paradoxically results in thrombosis and not in bleeding. Platelet factor 4 (PF4) released from platelet α-granules forms immunogenic PF4/heparin complexes. (*Marginal zone*) B lymphocytes generate IgG that recognize the PF4/heparin complexes; the Fc-parts of the IgG bind to platelet FcγIIa receptors, resulting in Fc receptor clustering and consequent strong platelet activation and aggregation. Platelet-derived microparticles that accelerate thrombin generation are produced. In addition, HIT antibodies directly or indirectly activate endothelial cells, and can also activate monocytes, resulting in additional tissue factor expression. Resulting increased thrombin generation leads to increased risk for thrombosis in HIT, providing a rationale for treatment that reduces thrombin generation. *Right side*: PF4 also binds to negatively charged molecules on bacteria, forming PF4/polyanion complexes, which express the same neoepitopes as on PF4/heparin complexes. PF4-coated bacteria also trigger PF4/polyanion antibody production. By the resulting antibodies, bacteria are opsonized and phagocytosed from granulocytes. The evolutionary advantage of this immune defense mechanism is that PF4 binds to different bacteria and PF4/polyanion antibodies can thereby bind to and opsonize pathogens the immune system has not seen before. Misdirection of this bacterial host-defense mechanism, in which heparin-coated platelets mimic bacteria, may underlie the development of HIT. Taken with modifications from Greinacher A, New England Journal Medicine 2015 (Greinacher 2015)

pentasaccharide, fondaparinux (Rauova et al. 2005). Nevertheless, fondaparinux can bind to PF4 (Greinacher et al. 2006) and induces anti-PF4/heparin antibodies to a similar extent as LMWH (Warkentin et al. 2005a). Potentially, fondaparinux interacts with PF4 synergistically with endothelial cell heparan sulfate or platelet membrane chondroitin sulfate, thereby inducing the immunogenic antigen. However, as multimolecular PF4/fondaparinux complexes do not form, fondaparinux only rarely causes HIT.

PF4 has been crystallized with fondaparinux and the Fab fragments of the HIT-antibody like monoclonal antibody KKO. The information obtained from these complexes further indicates that PF4 undergoes a certain structural change after binding to polyanions (Cai et al. 2015).

Other polyanions can also bind PF4 and induce the same conformational changes (Visentin et al. 1997; Greinacher et al. 1992). Especially relevant are PF4 interactions with nucleic acids (DNA, RNA) (Jaax et al. 2013). First, this could explain spontaneous HIT syndrome after knee replacement surgery (RNA release) (Warkentin et al. 2014). Second, nucleic acid-based drugs (aptamers, siRNAs) can bind to PF4 and induce thrombocytopenia, although it remains unresolved whether this reflects direct platelet activation by nucleic acids (Flierl et al. 2015) or in some situations a secondary immune mechanism.

Other Autoantigens Besides PF4 In virtually all patients with HIT, anti-PF4/heparin antibodies are detected. It has been suggested that non-PF4 antigens, such as interleukin-8 (IL-8) or neutrophil-activating peptide 2 (NAP-2), could be also involved (Amiral et al. 1996a; Regnault et al. 2003), but this remains unproven. Unlike anti-PF4/heparin antibodies, binding of anti-IL-8 and anti-NAP-2 antibodies to their respective antigens occurs even without heparin, but is enhanced by heparin. These antibodies tend to occur in patients with autoimmunity, cancer, infection, and trauma.

HIT as Misdirected Host Pathogen Defense Mechanism (Fig. 3) PF4 has a role in labeling pathogens for the immune system, as it binds in a charge-dependent fashion to gram-positive and gram-negative bacteria. Hereby, PF4 exposes the same antigenic complexes on bacteria as are formed with heparin on the platelet surface (Krauel et al. 2011). In a mouse model of *polymicrobial* bacterial sepsis, mice generated PF4/heparin-reactive antibodies (Krauel et al. 2011) without addition of heparin. Furthermore, an association between periodontal disease (frequently associated with bacteremia) and anti-PF4/heparin antibodies has been observed (Greinacher et al. 2011). Due to the ability of PF4 to bind to anionic cell walls of diverse bacterial species, anti-PF4/polyanion antibodies can even bind to bacteria not previously encountered by the host immune system.

Heparin-dependent antibodies are polyclonal and bind to multiple epitopes on PF4/heparin complexes (Li et al. 2002; Ziporen et al. 1998). Not all antibodies are able to activate platelets, however, and those features characterizing "pathogenic" (platelet-activating) antibodies remain largely uncertain. Platelet activation by anti-PF4/polyanion antibodies depends on the Fc part of the antibodies and can only be mediated by IgG, which bind through their Fc moieties to platelet FcγIIa receptors (FcγRIIa) (Kelton et al. 1988; Chong et al. 1989; Newman and Chong 2000). Cross-linking of the Fc receptors triggers platelet activation, release of platelet granules (containing PF4) and formation of platelet-derived microparticles, and ultimately platelet aggregation (Fig. 3) (Chong et al. 1981, 1982; Warkentin et al. 1994a). IgM antibodies might cause thrombocytopenia (although this is unproven), but most studies indicate they lack procoagulant effects (Juhl et al. 2006; Lindhoff-Last et al. 2001; Greinacher et al. 2007; Warkentin 2003; Amiral et al. 1996b). Some subclasses of anti-PF4/heparin IgG are more common ($IgG_1 > IgG_3 > IgG_2 > IgG_4$) (Amiral et al. 1996c). For induction of HIT, the quantity of IgG antibodies is more important than their subclass. Once the process of HIT-antibody-induced platelet activation begins, more PF4/heparin complexes are formed and bind to the platelet surfaces, thereby allowing more heparin-dependent antibodies to bind (Newman and Chong 2000). This self-enhancing chain reaction produces intense platelet activation (Chong et al. 1981). Generation of platelet-derived microparticles and other procoagulant materials leads to activation of blood coagulation pathways, thrombin generation, and thrombus formation ("hypercoagulability" of HIT) (Warkentin et al. 1994a; Warkentin 1996b).

Role of Endothelial Cells and Monocytes

Anti-PF4/polyanion antibodies also recognize PF4 bound to endothelial cell heparan sulfate, causing immunoinjury (Cines et al. 1987; Visentin et al. 1994). This occurs independent of FcγRIIa and exogenous heparin (Cines et al. 1987; Visentin et al. 1994). As human PF4 (hPF4) transgenic mice (expressing hPF4 on their endothelial cells, but lacking FcγRIIa receptors on their platelets) do not develop thrombosis after anti-hPF4/heparin antibodies are administered, direct endothelial cell activation alone is likely not a major mechanism of thrombosis in HIT. However, anti-PF4/polyanion antibodies also bind to PF4/thrombomodulin, thereby inhibiting activation of protein C, further increasing prothrombotic imbalance in HIT (Kowalska et al. 2011).

Anti-PF4/polyanion antibodies also bind to PF4/proteoglycan (chrondroitin sulfate) complexes on monocytes (Rauova et al. 2010), inducing monocyte activation via FcγRI (Kasthuri et al. 2012), resulting in expression of tissue factor and enhanced procoagulant activity (Arepally and Mayer 2001).

FcγRIIA Polymorphism

The only Fc receptor expressed by platelets is FcγRIIA, which has an Arg/His_{131} polymorphism. The role of this polymorphism as a risk factor for HIT is controversial (Brandt et al. 1995; Burgess et al. 1995; Denomme et al. 1997; Arepally et al. 1997; Bachelot-Loza et al. 1998; Carlsson et al. 1998). Two major studies are consistent, however, in showing FcγRIIA 131RR increases risk for thrombosis in HIT. One was a large cohort study (Carlsson et al. 1998), and the other a combination of in vitro studies and a cohort study (Rollin et al. 2015) showing that due to the lower affinity of FcγRIIA 131RR for monomeric IgG (especially IgG_2), the threshold for platelet activation by PF4/polyanion/IgG immune complexes is lower (because monomeric IgG cannot block the receptor).

Immune Response

The immune response of HIT has several atypical features. Anti-PF4/polyanion IgG antibodies are generated as early as 4 days (median, day 6) (Greinacher et al. 2005a; Selleng et al. 2010; Pouplard et al. 1999; Warkentin et al. 2000), even in patients receiving heparin for the first time. Further, IgM and IgA antibodies can be generated contemporaneously (i.e., without IgM precedence), and with IgG being the predominant class of antibodies formed (Greinacher et al. 2009; Warkentin et al. 2009b). Further, antibody titers decrease rapidly within several weeks (Warkentin and Kelton 2001b; Greinacher et al. 2009), which is also unusual for a secondary immune response.

Increasingly, "spontaneous HIT syndrome" (Warkentin et al. 2014) is observed in which patients develop an illness

clinically and serologically mimicking HIT despite no proximate heparin exposure. Patient serum contains strongly reacting anti-PF4/heparin IgG with autoantibody-like features, which recognize platelet-bound PF4 in the absence of pharmacologic heparin (Warkentin and Kelton 2001a; Socher et al. 2008; Prechel et al. 2005; Padmanabhan et al. 2015a). The history of recent bacterial infection or knee replacement surgery points to a pathogenesis involving PF4/bacteria or PF4/nucleotide complexes that induce primary immunization and, when boosted, (rarely) spontaneous HIT.

In mouse models, the immune response after active immunization with PF4/heparin complexes is mediated by marginal zone B-cells (Zheng et al. 2013) and is dependent on the presence of T-cells (Zheng et al. 2015). However, human and murine B-cells can also be stimulated to produce anti-PF4/polyanion antibodies by in vitro stimulation with proinflammatory molecules, such as CpG. Furthermore, mice lacking protein kinase Cδ (PKCδ) that are prone to breakdown of B-cell tolerance produce anti-PF4/heparin antibodies spontaneously. Therefore, breakdown of B-cell tolerance may play a role in HIT pathogenesis (Zheng et al. 2014). Monocytes take up PF4/heparin complexes by an active endocytic pathway and then transport them to late endosomes, where the complexes still express the antigen recognized by HIT antibodies. This raises the intriguing hypothesis that PF4/heparin complexes are seen by the immune system as particulate antigens. Such antigens are especially immunogenic, as they are more resistant to proteolysis and can, in some instances, be recycled to the cell surface, allowing for antigen persistence (Joglekar et al. 2015).

Frequency of HIT

HIT occurrence has several requirements (Warkentin 2011). First, heparin exposure triggers a humoral immune response. This usually occurs when heparin is given in a setting of platelet activation and inflammation (e.g., intra- or perioperatively). Second, the "HIT antibodies" must be able to induce platelet activation in vivo (sufficient plasma levels of platelet-activating IgG antibodies). Finally, these events must occur in a susceptible host (platelets become activated). Therefore, the incidence of HIT varies among different patient populations (Fig. 4 (Greinacher and Warkentin 2008)).

The overall frequency of (immune) HIT in hospitalized patients who receive heparin is ~0.2 % (Smythe et al. 2007), but this includes brief or trivial heparin exposures. Some patient populations have frequencies of HIT as high as ~5 % (e.g., post-orthopedic surgery females receiving prophylactic-dose UFH for at least 10 days (Greinacher et al. 2005a)) or even 10 % (post-ventricular assist device implantation receiving therapeutic-dose UFH) (Warkentin et al. 2009a). Risk factors for HIT include heparin type (UFH > LMWH > fondaparinux), patient type (surgical [major > minor trauma] > medical > obstetrical/pediatric), sex (female > male), and dose and duration of heparin exposure (Warkentin et al. 1995, 2006, 2011a; Lubenow et al. 2010; Linkins and Lee 2013).

The risk of HIT is approximately tenfold less with LMWH versus UFH (Warkentin et al. 2006; Martel et al. 2005), reflecting a lower rate of antibody formation as well as a reduced frequency of thrombocytopenia "breakthrough" among immunized patients for LMWH versus UFH (Warkentin et al. 2013). The frequency of HIT with

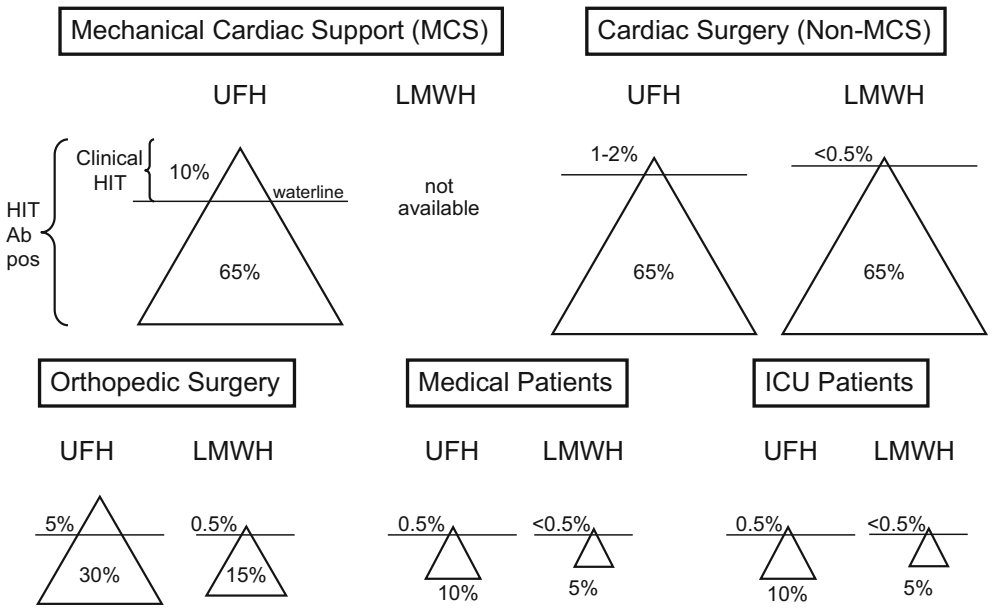

Fig. 4 Multiple iceberg model. The frequency of HIT in different patient populations treated with UFH versus LMWH is shown. The size of the iceberg reflects relative frequencies of anti-PF4/heparin formation, whereas the iceberg tip protruding above the waterline indicates the relative frequency of breakthrough of HIT. Modified from: Greinacher A, Warkentin TE. Risk of heparin-induced thrombocytopenia in patients receiving thromboprophylaxis. Exp Rev Hematol 2008; 1 (1): 75–85 (Greinacher and Warkentin 2008)

fondaparinux (sulfated pentasaccharide) appears negligible (but not zero) (Warkentin et al. 2005a, 2011a).

Anti-PF4/heparin antibodies first become detectable about 4 days (median) after starting an immunizing exposure to heparin, with antibody levels peaking approximately a week later (Greinacher et al. 2009; Warkentin et al. 2009b). Thus, greater duration of heparin (up to about 10 days) increases risk of HIT, with later occurrences (despite prolonged administration) uncommon.

The role of heparin "flushes" or device-bonded heparin in triggering or maintaining HIT is controversial. For example, onset of HIT in a patient receiving heparin flushes or an implanted heparin-bonded device could reflect immunization due to earlier intraoperative administration of UFH as well as the formation of highly pathogenic HIT antibodies that do not require further heparin to cause thrombocytopenia (Warkentin 2013b).

The frequency of HIT is higher if the definition of thrombocytopenia requires a proportional (relative) platelet count fall (e.g., >50 %), rather than a drop below an arbitrary threshold (e.g., 150×10^9/L), as the former definition better accounts for the phenomenon of postoperative thrombocytosis (Warkentin et al. 2003).

HIT is less common in medical than surgical patients (Warkentin et al. 2006), perhaps reflecting lack of perioperative PF4 release as an immunizing trigger. In critically ill patients, HIT is a relatively uncommon cause for thrombocytopenia: less than 1 % of such patients have HIT as the explanation for thrombocytopenia (PROTECT Investigators for the Canadian Critical Care Trials Group and the Australian and New Zealand Intensive Care Society Clinical Trials Group et al. 2011; Selleng et al. 2007; Warkentin 2015b). Indeed, the high frequency of subclinical seroconversion among heparin-treated patients means that early-onset and persisting thrombocytopenia in the intensive care unit rarely indicates HIT even when HIT immunoassays are positive (Selleng et al. 2010).

For unknown reasons, HIT is rare in pregnant women and in children, and does not appear to occur in neonates (Klenner et al. 2004).

The "stoichiometric" requirements for optimal PF4/heparin ratios in triggering immunization could help explain why HIT risk is higher with greater magnitude of trauma (Lubenow et al. 2010) and perhaps also why greater body mass index (BMI) was associated with a greater risk of anti-PF4/heparin antibody formation (since higher BMI predicts for more optimal PF4/heparin ratios when fixed-dose postoperative heparin thromboprophylaxis is given) (Warkentin et al. 2010).

Diagnosis

Clinical Diagnosis

HIT should be suspected whenever an otherwise unexplained drop in platelet count and/or thrombosis *begins* on/after day 5 of heparin administration, usually by day 10 (first day of heparin use = day 0). This is called "typical-onset HIT." The diagnosis should also be considered in patients with unexpected thrombocytopenia, especially when accompanied by new thrombosis that occurs (or worsens) after stopping heparin ("delayed-onset" or "autoimmune-like" HIT). Also, when heparin administration is associated with an abrupt and otherwise unexplained platelet count fall, "rapid-onset HIT" should be considered if patients have received a (presumably immunizing) recent exposure to heparin (see Fig. 1 shown earlier in the chapter).

A clinical scoring system, the "4Ts" (Table 4), is based on the mnemonic, *T*hrombocytopenia, *T*iming, *T*hrombosis (or other sequelae of HIT), and (lack of) o*T*her explanations for thrombocytopenia (Lo et al. 2006) A more recent scoring system is the HIT Expert Probability (HEP) score (Cuker et al. 2010). Scores are helpful in ruling out HIT, because a low score suggests a low (<2 %) probability of having a positive

Table 4 4Ts scoring system for HIT

4Ts category	2 points	1 point	0 point
*T*hrombocytopenia	Platelet count fall >50 % and platelet count nadir ≥20	Platelet count fall 30–50 % or platelet count nadir 10–19 (maximum, 1 point, for >50 % platelet count fall that directly results from surgery)	Platelet count fall <30 % or platelet count nadir <10
*T*iming (of onset of platelet count fall)	Clear onset day 5–10 or platelet fall ≤1 day (prior heparin exposure within 30 days)	Consistent with day 5–10 fall, but not clear (e.g., missing platelet counts); onset after day 10; or fall ≤1 day (prior heparin exposure 30–100 days ago)	Platelet count fall ≤4 days without recent heparin exposure
*T*hrombosis (or other sequelae)	New thrombosis (confirmed); necrotizing skin lesion(s) at heparin injection site(s); post-heparin anaphylactoid reaction	Progressive or recurrent thrombosis; non-necrotizing (erythematous) skin lesions at heparin injection sites; suspected thrombosis (not proven)	None
o*T*her causes of thrombocytopenia	None apparent	Possible	Definite

The 4Ts score is the sum of the values for each of the four categories. Scores of 1–3, 4–5, and 6–8 correspond to low, intermediate, and high probability of HIT, respectively

platelet activation assay; however, even a high score suggests a probability of HIT of only ~50 % (Cuker et al. 2012).

Laboratory Investigations

HIT can be difficult to exclude or confirm based on clinical information alone. Laboratory testing for heparin-dependent antibodies is therefore a mainstay of diagnosis. Two classes of assays are available: functional (platelet activation) assays and (PF4-dependent) immunoassays (Table 5). In general, testing should only be performed when HIT is reasonably suspected (e.g., 4Ts score of 4 points or greater, Table 4), given their low diagnostic specificity, especially for the immunoassays (Greinacher et al. 2007).

Functional Assays

The presence of platelet-activating antibodies can be established only by using functional assays. There are

Table 5 Laboratory tests for HIT

Test system	Principle	Detected antibodies	Sensitivity	Specificity	NPV	PPV	Advantages	Drawbacks
PaGIA	Beads, gel centrifugation	Polyspecific	91–94	81–88	99.5–99.1	35.1–36.6	One sample, short turnaround time	Visual interpretation, Difficult to quantify
HealthTEST	Beads, membrane filter	Polyspecific	Non-informative	30–35			One sample, easy to perform, no special equipment	Non-informative
LFI-HIT Melinea	Beads, lateral flow technology	IgG	Visual: 100 Scanner: 97	93 93	100 99.7	54.0 52.4	One sample, short turnaround time, no special equipment	Serum samples are required
HemosIL® HIT-Ab	Beads, agglutination	Polyspecific	100	76	100	45.5	Automated and quantifiable, standardization makes results comparable between laboratories	
HemosIL® AcuStar HIT-Ab	Beads, chemiluminescent detection	Polyspecific	98	82	99.7	41.8	Automated and quantifiable, standardization makes results comparable between laboratories	
HemosIL® AcuStar HIT-IgG	Beads, chemiluminescent detection	IgG	96.2	96.5	99.5	78.1	Automated and quantifiable, standardization makes results comparable between laboratories	
Stago Diagnostica	Enzyme-linked immunosorbent assays	IgG (polyspecific)	98	90				Time and cost intensive
Immucor GTI Diagnostics	Enzyme-linked immunosorbent assays	IgG (polyspecific)	100 100	89 81	100 100	43 28		Time and cost intensive
HYPHEN BioMed	Enzyme-linked immunosorbent assays	IgG (polyspecific)	100	87	100	39	Detects heparin-dependent antigens other than PF4	Time and cost intensive
In-house ELISAs	Enzyme-linked immunosorbent assays	Separately IgG, IgM, IgA	98–100	86–88	99.7	47.2	Require in-house quality control measures	

Althaus et al. (2013), Arepally et al. (1995), Bakchoul et al. (2009, 2011), Lindhoff-Last et al. (2001), Morel-Kopp et al. (2012), Pouplard et al. (2007), Sachs et al. (2011), Warkentin et al. (2007), Whitlatch et al. (2008)

(1) assays that use whole blood or platelet-rich plasma (PRP) and (2) assays that use washed platelets.

Whole Blood Assay

The whole blood impedance analyzer (Multiplate®, multiple electrode platelet aggregometry) detects platelet activation in the presence of heparin and patient plasma. Blood from a selected donor is collected in hirudin-containing tubes. UFH is then added (0.5 or 100 IU/mL final concentration) and the suspension incubated with patient-citrated platelet-poor plasma (PPP) or heat-inactivated serum. Changes in impedance are then recorded over a 15 min period. This assay, which does not require platelet preparation, may have similar sensitivity as the serotonin-release assay (SRA) when performed using blood from a donor whose platelets are known to react well to HIT antibodies (Morel-Kopp et al. 2012).

Platelet-Rich Plasma Assay

In the platelet aggregation test (PAT), platelet aggregometry is performed with citrated PRP isolated from one selected donor. Platelet activation (aggregation) is investigated in the presence of patient PPP (or serum) and heparin in two concentrations (0.5 and 100 IU/mL). Although this assay is easier to perform compared with washed platelet assays, its sensitivity is inferior to the SRA even when good platelet donors are selected (Chong et al. 1993).

Washed Platelet Assays

In the heparin-induced platelet activation (HIPA) assay and the serotonin-release assay (SRA) (Greinacher et al. 1991; Warkentin et al. 1992), washed platelets are used to investigate platelet activation by heparin-dependent antibodies, either by visually observing formation of platelet aggregates or measuring release of radiolabeled serotonin, respectively.

In the HIPA assay, washed platelets from four healthy unselected donors are used. Washed platelets are incubated with patient serum in the presence of buffer or heparin (0.2 and 100 IU/mL). Incubation takes place in a round-bottom microtiter plate, with spinning magnetic spheres used as source of shear force. Platelet aggregate formation is determined visually at 5-min intervals; the test is positive if aggregation is observed within 30 min (at 0.2 but not at 100 IU/mL heparin) using the platelet suspensions of least two out of four donors.

In the SRA, platelets obtained from a selected donor are preincubated with radioactive ^{14}C-serotonin. After washing, platelets are incubated with patient serum and heparin in flat-bottomed microtiter wells in duplicate on a plate shaker. After incubation for 60 min and centrifugation, supernatants of each reaction mixture are collected and radioactivity measured. Test results are expressed as percentage of serotonin release (compared to 100 % value determined by detergent-induced platelet lysis). The test is considered positive if there is >20 % release at low heparin concentrations (0.1–0.3 IU/mL) and <20 % release at supratherapeutic heparin (100 IU/mL) levels (although sera from patients with clinical HIT usually cause >50 % serotonin release (Warkentin et al. 2013)).

Both functional assays are considered the "gold standard" for diagnosing HIT. However, these assays are difficult to perform, require selected healthy platelet donors, and are restricted to few reference laboratories. Moreover, the SRA requires the use of the radioisotope, [^{14}C]-serotonin, which most laboratories try to avoid due to regulatory and safety issues.

PF4-Dependent Immunoassays

Antibody binding can be detected by enzyme-linked immunosorbent assays (ELISAs) and particle-based immunoassays.

In ELISAs, the target antigen (PF4/polyanion complex) is bound onto a solid phase, e.g., microtiter plate wells. Patient serum or plasma is added, and an enzyme-labeled secondary antibody is used to detect anti-PF4/heparin antibodies binding in a semiquantitative fashion. The intensity of color change, measured as optical density (OD), is proportional to the amount of bound antibodies.

At least three ELISAs are commercially available. They differ in source of PF4 and type of polyanion: (1) Diagnostica Stago (Asnières-sur-Seine, France) utilizes recombinant PF4 bound to heparin (Arepally et al. 1995); (2) Immucor GTI Diagnostics (Waukesha, WI, USA) makes use of purified PF4 (obtained from outdated platelets) bound to the non-heparin polyanion, polyvinyl sulfonate (Visentin et al. 1994); and (3) HYPHEN BioMed (Neuville-sur-Oise, France) uses platelet lysate (source of PF4 and, possibly, other heparin-binding proteins) to bind to surface-immobilized heparin.

Although ELISAs have an excellent negative predictive value (NPV) to rule out HIT, their specificity is low (40–80 %, depending on the assay). A minor drawback of most immunoassays is that they only detect PF4/polyanion-dependent antibodies, but whether the ELISA that uses platelet lysate has advantages is uncertain (Regnault et al. 2003; Bounameaux et al. 2007).

Particle-based immunoassays detect anti-PF4/heparin antibodies through agglutination of particles coated with PF4/heparin complexes. Several assays are available. In the particle gel immunoassay (PaGIA, DiaMed division of Bio-Rad Laboratories, Cressier sur Morat, Switzerland), red-colored, agglutinated beads are unable to migrate through the sephacryl gel, which is detected visually (Meyer et al. 1999). The major advantage of the PaGIA is its rapid turnaround time (15 min). Although occasional

false-negative test results have been reported (Bakchoul et al. 2009; Pouplard et al. 2007), a recent prospective management study showed high NPV even when the pretest probability of HIT seemed high (Linkins et al. 2015).

Another rapid assay is the "HealthTEST Heparin/Platelet factor 4 Antibody Assay" (Akers Biosciences, Inc., Thorofare, NJ, USA). Here, a membrane filter is used to separate non-agglutinated blue-colored particles from agglutinated ones. However, this assay fared poorly in comparative studies (Warkentin et al. 2007).

Recently, lateral flow technology was used as a point-of-care test for anti-PF4/heparin antibodies (Sachs et al. 2011). HIT antibodies are detected using the principle of capillary action which induces a flow of the test sample along a solid phase. This test system is designed to investigate one patient sample and can be performed in <15 min without the need for special equipment. Interestingly, not only a high NPV but also a satisfactory positive predictive value (PPV) was found.

Automated immunoassays have been recently introduced: one is based on the agglutination of latex particles (HemosIL® HIT-Ab (PF4-H)) and two are based on chemiluminescent detection system (HemosIL® AcuStar HIT-Ab (PF4-H) and HemosIL® AcuStar HIT-IgG (PF4-H), respectively). In the latex agglutination assay, the (expected) agglutination of PF4/polyvinyl sulfonate-coated beads by a monoclonal anti-PF4/heparin antibody is inhibited in the presence of anti-PF4/heparin antibodies (Legnani et al. 2010). These assays have high NPV and informative PPVs (Althaus et al. 2013).

Strategies to Reduce Overdiagnosis

Most laboratories perform immunoassays in their algorithm to diagnose HIT. However, as few as 10–15 % of sera from patients suspected for HIT, and only up to 50 %, of ELISA-positive patient sera additionally contain the clinically relevant, platelet-activating antibodies characteristic of HIT. Several approaches may increase the diagnostic specificity of ELISAs: selective detection of IgG antibodies, analyzing the magnitude of the OD, and use of a high heparin inhibition step.

Exclusive Detection of IgG

Detection of anti-PF4/heparin antibodies of IgM and IgA classes reduces diagnostic specificity of immunoassays (Lindhoff-Last et al. 2001; Warkentin et al. 2005b), and exclusive detection of anti-PF4/heparin IgG improves operating characteristics without loss of sensitivity (Bakchoul et al. 2009) provided that the manufacturer's recommended cutoff is used (Nagler et al. 2015).

Optical Density

The magnitude of the OD value corresponds strongly with the likelihood of a positive functional test and hence a diagnosis of HIT (Warkentin 2008; Greinacher et al. 2010). Weak positive test results (OD <1.0), especially with a polyspecific ELISA (i.e., detecting IgG, IgA, and/or IgM), are in fact strong evidence *against* the diagnosis of HIT. Laboratory cutoffs should be individualized, however, because OD values are arbitrary units and can differ considerably between photometers (Greinacher et al. 2010).

High Heparin Inhibition Step

The specificity of HIT immunoassays can be improved by the implementation of a confirmation step using supratherapeutic concentrations of heparin. This is because a persistently positive test despite high heparin concentrations can indicate an antibody that reacts against PF4 but not to the PF4/heparin complex. Such antibodies usually do not indicate HIT. While some studies support the use of this step (Warkentin et al. 2008b; Whitlatch et al. 2008), especially for weakly positive OD values <1.0 units (Althaus et al. 2011, 2013), some of the clinically most relevant high-titer antibodies with strong platelet-activating capacity are not inhibited (Warkentin et al. 2008b; Bakchoul et al. 2011). A recent meta-analysis did not find this maneuver to be helpful (Nagler et al. 2015).

Suggested Algorithm to Diagnose HIT

Diagnostic algorithms that combine clinical features and results of laboratory testing are available for diagnosis of HIT (Fig. 5). Screening PF4-dependent immunoassays are indicated for patients with at least intermediate probability of HIT. If the ELISA is positive, a functional assay should be also performed to confirm or refute a diagnosis of HIT (Warkentin et al. 2011b).

Future Assays

A transgenic B-cell line expressing FcγRIIa coupled to a luciferase reporter, to which patient serum and PF4/heparin is added, can detect platelet-activating IgG that bind to PF4/heparin complexes on the B-cells, thereby cross-linking the FcγRIIa, activating the coupled luciferase, and producing a luminescence signal (Cuker et al. 2013). The other

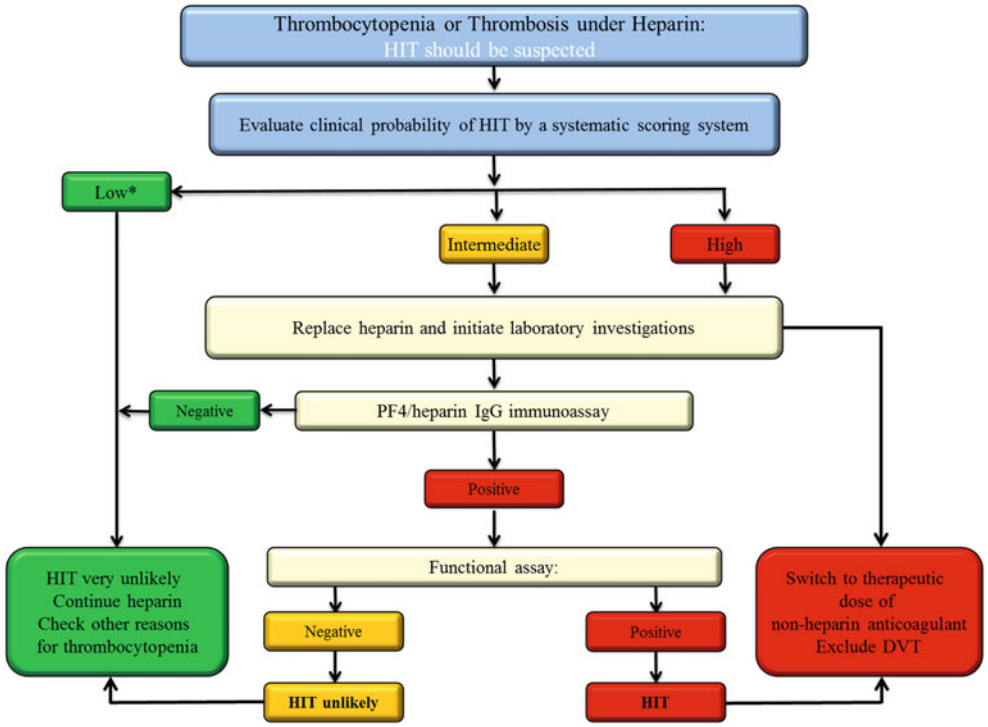

Fig. 5 Diagnostic flow chart for HIT. The flow chart provides a guide on decision making in a patient suspected to have HIT. Antigen assays for PF4/heparin antibodies are widely available, while functional assays using washed platelets such as the serotonin-release assay or the HIPA test are restricted to specialized laboratories. In centers where a functional assay is unavailable or cannot be obtained promptly, other options include using high reactivity of the antigen test (e.g., an OD >1.0) as a surrogate marker for clinically relevant antibodies or incorporating the 4T score in interpretation (indicated by the *gray*, *hatched arrows*), although overdiagnosis of HIT remains possible. Even if acute management decisions are made without confirmation of platelet-activating, heparin-dependent antibodies, effort should be made to rule out or confirm the presence of these antibodies to guide future patient management

assay measures proteolysis of FcγRIIa (Nazi et al. 2013). Both assays require further evaluation.

Recently, it has been suggested that supplementation of PF4 to functional assays increases sensitivity for detecting platelet-activating antibodies (Nazi et al. 2015; Padmanabhan et al. 2015a, b), but whether this improves overall diagnostic performance is unclear.

HIT Management

Treatment of suspected HIT involves cessation of heparin (including low-molecular-weight heparin, heparin flushes, and heparin-bonded catheters) and initiation of a parenteral non-heparin anticoagulant. Laboratory studies should be ordered to confirm the diagnosis of HIT, but treatment should not be withheld pending results if HIT is strongly suspected. Our suggestions for management of HIT are summarized in Table 6 and described in detail below.

Parenteral Anticoagulants for the Treatment of HIT

Parenteral anticoagulants for use in treating HIT are detailed below. The mechanism of action, dosing, monitoring, and metabolism of these agents are summarized in Table 7. Choice of anticoagulant should be based on the patient's hepatic and renal function, clinical stability, drug availability, and physician experience.

Argatroban

Argatroban is a hepatically metabolized direct inhibitor of thrombin. It is the only FDA-approved agent for the treatment of HIT available in the USA. The pivotal studies leading to its approval (ARG-911 and ARG-915) were single-arm open-label studies in which argatroban-treated subjects were compared to historical untreated controls (Lewis et al. 2001, 2003). In a pooled analysis of ARG-911 and ARG-915, argatroban reduced the risk of new thrombosis by approximately 70 % compared with controls. The rate of major bleeding was 0.99 %/day (Lewis et al. 2006). A shortcoming of these studies is that serologic confirmation of HIT was not required for enrollment (but was required for the control subjects). Post hoc laboratory testing

demonstrated that approximately one-third of subjects were antibody-negative and likely did not have HIT, a limitation that may have biased the results in favor of argatroban (Walenga et al. 1999). Argatroban is administered as a continuous intravenous infusion. Treatment is monitored with the APTT or, less commonly, the dilute thrombin time or ecarin clotting time. Dose reduction is advised in patients with hepatic dysfunction, heart failure, anasarca, and after cardiac surgery (Table 7) (Linkins et al. 2012).

Bivalirudin

Bivalirudin, another direct thrombin inhibitor, is less reliant on liver and kidney function for clearance and has a shorter half-life than argatroban (25 vs. 40–50 min). Thus, it is a potentially attractive option for situations where rapid dose titration is desired, e.g., cardiac surgery, percutaneous coronary intervention (PCI), and critical illness with multiorgan failure. Bivalirudin has been studied for PCI in large clinical trials and is approved for anticoagulation during PCI for both HIT and routine (non-HIT) settings (Lehman and Chew 2006). Protocols have also been established for patients undergoing cardiac surgery with or without cardiopulmonary bypass (Dyke et al. 2006; Koster et al. 2006). Assessment of bivalirudin in hospitalized patients, including patients with combined hepatic and renal dysfunction, is limited to retrospective studies (Kiser and Fish 2006; Joseph et al. 2014). Bivalirudin is administered as a continuous intravenous infusion. In the operating room and catheterization suite, it is monitored by activated clotting time. Elsewhere, the APTT is used (Table 7).

Danaparoid

Danaparoid is an antithrombin-dependent inhibitor of factor Xa; inhibition of thrombin is also mediated by heparin cofactor II. It is approved for the treatment of HIT in multiple jurisdictions, but not in the USA, where it has been withdrawn from the marketplace. In an open-label trial, patients with HIT and thrombosis were randomized to danaparoid or dextran-70. Recovery from thrombosis was greater in the danaparoid arm (56 % vs. 14 %, $p = 0.02$) (Chong et al. 2001). Danaparoid is given intravenously as a bolus followed by accelerated initial infusion and then maintenance infusion (Table 7). Low (prophylactic-)-dose danaparoid (e.g., 750 U twice or thrice daily by subcutaneous injection) should be avoided in patients with HIT because of a relatively high rate of breakthrough thrombosis (Farner et al. 2001). In vitro cross-reactivity of HIT antibodies with danaparoid occurs in a minority of patients, but is rarely clinically significant, and routine testing for in vitro cross-reactivity is not recommended (Magnani and Gallus 2006).

Fondaparinux

Fondaparinux, a synthetic pentasaccharide, is an antithrombin-dependent inhibitor of factor Xa. It is approved for the treatment and prevention of venous thromboembolism, but is not approved for treatment of HIT. However, it is widely used for treatment of HIT and appears to have similar efficacy and safety to approved agents (Warkentin et al. 2011c; Schindewolf et al. 2014; Kang et al. 2015). Although anti-PF4/heparin antibody formation is common in postoperative patients receiving thromboprophylaxis with fondaparinux, and a small number of cases of HIT possibly caused by fondaparinux in this setting have been reported, the risk of fondaparinux causing further thrombocytopenia when used to treat HIT appears to be negligible (Motokawa et al. 2011; Warkentin et al. 2011a). Fondaparinux is administered by once-daily subcutaneous injection, does not require routine monitoring (Table 7), and may therefore facilitate transition to the outpatient setting. Because of these advantages, we frequently use fondaparinux for management of stable patients with acceptable renal function.

Limitations of Approved Agents and Investigational Therapies

Argatroban and danaparoid, the only agents licensed for management of HIT, are associated with important limitations. Both drugs are expensive. Management is complex, involving continuous intravenous infusion, frequent laboratory monitoring, and dose adjustment. The therapeutic indices for these agents are narrow. They carry a ~1 % daily risk of major hemorrhage and do not reduce frequency of limb amputation or death (Lewis et al. 2006; Hursting and Verme-Gibboney 2008; Chong et al. 2001; Magnani and Gallus 2006). The availability of danaparoid has been limited by worldwide shortages. Monitoring of argatroban by APTT may be confounded by HIT-associated consumptive coagulopathy and result in underdosing (Warkentin 2014). Argatroban also raises the international normalized ratio (INR), complicating transition to VKAs (Sheth et al. 2001).

Fondaparinux greatly reduces the complexity of HIT management. It does not require routine monitoring or dose adjustment, is administered subcutaneously, and has a negligible effect on the INR, thereby facilitating transition to outpatient therapy. Direct oral inhibitors of thrombin (e.g., dabigatran) and factor Xa (e.g., rivaroxaban, apixaban, edoxaban) do not cross-react with HIT antibodies (Krauel et al. 2012) and carry similar practical advantages, but published experience with these agents in patients with HIT is limited (Linkins et al. 2014), and low trough levels may not provide adequate protection for highly prothrombotic states such as HIT. Accordingly, use is not recommended outside of a clinical trial (Linkins et al. 2016). Other novel therapies for HIT including PF4 antagonists, FcγRIIA blockers, and splenic tyrosine kinase inhibitors are in preclinical development (Cuker 2012).

Transitioning to Oral Anticoagulants

Patients with HIT who are treated with a VKA are at risk for venous limb gangrene, a potentially catastrophic thrombotic

Table 6 Summary of suggestions for management of patients with acute HIT

Topic	Suggestion(s)
Parenteral anticoagulation	• In patient with strongly suspected or confirmed acute HIT, heparin should be discontinued, and a parenteral non-heparin anticoagulant (e.g., argatroban, bivalirudin, danaparoid, fondaparinux) should be initiated in therapeutic intensity • We prefer fondaparinux in stable patients with acceptable renal function
Screening for lower extremity deep-vein thrombosis	• We request compression ultrasonography of the bilateral lower extremities in patients with isolated HIT to screen for silent deep-vein thrombosis
Platelet transfusion	• We recommend avoidance of routine prophylactic platelet transfusion in patients with suspected or confirmed HIT • Platelet transfusion may be considered in patients with or at risk for serious bleeding
Transition to oral anticoagulation	• Patients may be transitioned to an oral anticoagulant after the platelet count recovers to a stable plateau • If a VKA is used, it should be overlapped with a parenteral anticoagulant for $\geq$5 days and until the INR reaches the intended target • If a DOAC is used, overlap with a parenteral anticoagulant is not required
Duration of anticoagulation	• In patients with HIT and thrombosis, we suggest 3–6 months of anticoagulation • In patients with autoimmune HIT and thrombosis, we suggest to continue anticoagulation until the platelet count recovers to a stable plateau and until the results of the functional assay revealed no further activation in the absence of heparin, but in case of thrombosis at least for 3–6 months • In patients with isolated HIT and negative compression ultrasonography of the lower extremities, we continue anticoagulation until the platelet count recovers to a stable plateau

Table 7 Parenteral anticoagulants for the treatment of HIT

Drug	Clearance (half-life)	Initial dosing	Monitoring
Direct thrombin inhibitors			
Argatroban	Hepatobiliary (40–50 min)	Bolus: none Continuous infusion: normal organ function → 2 μg/kg/min Liver dysfunction (bilirubin > 1.5 mg/dL) → 0.5–1.2 μg/kg/min Heart failure, anasarca, post-cardiac surgery → 0.5–1.2 μg/kg/min	Adjust to APTT 1.5–3.0 times baseline
Bivalirudin[a]	Enzymatic/renal (25 min)	Bolus: none Continuous infusion: normal organ function → 0.15 mg/kg/h Renal or liver dysfunction → dose reduction may be appropriate	Adjust to APTT 1.5–2.5 times baseline
Indirect factor Xa inhibitors			
Danaparoid	Renal (24 h)	Bolus: <60 kg → 1500 units 60–75 kg → 2250 units 75–90 kg → 3000 units >90 kg → 3750 units Accelerated initial infusion: 400 units/h × 4 h, then 300 units/h × 4 h Maintenance infusion: normal renal function → 200 units/h Renal dysfunction → 150 units/h The daily dose can also given as s.c. injection, after the acute phase of HIT, i.e., platelet counts normalized	Adjust to danaparoid-specific anti-Xa activity of 0.5–0.8 units/mL
Fondaparinux[a]	Renal (17–24 h)	<50 kg → 5 mg SC daily 50–100 kg → 7.5 mg SC daily >100 kg → 10 mg SC daily Cl_{Cr} 30–50 mL/min → use caution Cl_{Cr} <30 mL/min → avoid use	None[b]

APTT activated partial thromboplastin time, Cl_{Cr} creatinine clearance

[a]Not approved for treatment of HIT

[b]Some authorities monitor using fondaparinux-specific anti-Xa activity

complication of the microvasculature secondary to depletion of protein C (Warkentin et al. 1997). Thus, warfarin should be avoided in patients with HIT until the platelet count recovers to a stable plateau within the normal range. When a VKA is initiated, large loading doses (e.g., warfarin >5 mg/day) should be avoided, and the VKA should be overlapped with a parenteral anticoagulant for at least 5 days and until the INR has reached the intended target. If a patient happens to be on a VKA when HIT is newly diagnosed, it should be discontinued, reversed with vitamin K, while at the same time an alternative anticoagulant is started in therapeutic dose (Linkins et al. 2012).

Transitioning from argatroban to a VKA is challenging because argatroban prolongs the INR. Algorithms have been developed to guide this transition and avoid under-anticoagulation (Sheth et al. 2001).

Duration of Anticoagulation

As with other thromboembolic events secondary to a transient provoking factor, 3–6 months of anticoagulation is generally sufficient for patients with HIT and thrombosis. For patients with isolated HIT, the optimal duration of anticoagulation is controversial. In a historical series of 62 untreated patients with isolated HIT, the cumulative incidence of thromboembolism at 30 days was 52 %. The majority of events occurred within 10 days of heparin cessation, a period corresponding to platelet recovery (Warkentin and Kelton 1996). At a minimum, anticoagulation should therefore be continued in patients with isolated HIT until platelet recovery. Some experts recommend a longer course of treatment (4–6 weeks) (Linkins et al. 2012).

Also controversial is whether asymptomatic patients with HIT should undergo screening lower-limb compression ultrasonography. Silent DVT is common in patients with HIT (Tardy et al. 1999), but whether its identification should influence duration of anticoagulation is uncertain. We perform leg vein ultrasound in all patients with serologically confirmed isolated HIT and continue anticoagulation for at least 3 months in those with proximal DVT.

Platelet Transfusion

There is a theoretical concern that platelet transfusion may precipitate thrombosis in patients with HIT, though published evidence is conflicting (Goel et al. 2015; Hopkins and Goldfinger 2008; Refaai et al. 2010). Because HIT generally manifests as a prothrombotic rather than a hemorrhagic diathesis, prophylactic platelet transfusion is rarely indicated. However, transfusion may be appropriate in the setting of significant bleeding, high bleeding risk (e.g., during alternative anticoagulation with severe thrombocytopenia or an invasive procedure), or diagnostic uncertainty.

Vascular Access Devices and Inferior Vena Cava Filters

HIT-associated thrombosis demonstrates a propensity to occur at sites of vessel injury (Greinacher et al. 2005b; Hong et al. 2003). We therefore recommend against insertion of central venous catheters and arterial lines unless absolutely necessary. Inferior vena cava filters may have serious consequences including caval thrombosis and progression of DVT to ischemic limb gangrene (Ishibashi et al. 2005). Moreover, their use does not obviate the need for anticoagulation. Thus, they should generally be avoided in patients with HIT.

Heparin Reexposure for Cardiovascular Procedures and Hemodialysis in Patients with a History of HIT

HIT antibodies are transient and generally resolve within several weeks or a few months in a predictable sequence: functional assays become negative first (median, 50 days post-heparin cessation), followed by immunoassays (median, 80 days) (Warkentin and Kelton 2001b). HIT laboratory testing will determine where a patient is along this sequence, guiding timing of heparin reexposure, if required (e.g., intraoperative anticoagulation for cardiovascular surgery) (Table 8).

The safety of intraoperative heparin in remote HIT (negative functional and immunologic assays) was first

Table 8 Recommendations for intraoperative anticoagulation in patients with a history of HIT

Phase	Functional assay	Immunoassay	Recommendation
Acute	+	+	1. Delay surgery 2. If surgery cannot be delayed, use an alternative anticoagulant (e.g., bivalirudin), or treat with preoperative plasma exchange until functional assay becomes negative
Subacute[a]	–	+	1. Delay surgery 2. If surgery cannot be delayed, consider heparin
Remote	–	–	1. Heparin

[a]Defined as patients with recent HIT whose platelet count has recovered but who remain antibody-positive; heparin reexposure can be considered if the functional test is negative even if the PF4-dependent immunoassay is still positive

established in a series of ten patients undergoing cardiac surgery. None developed recurrent HIT or recrudescence of HIT antibodies (Potzsch et al. 2000). Intraoperative anticoagulation in patients with subacute HIT (negative functional assay, positive immunoassay) is more controversial. In three patients with subacute HIT who required urgent heart transplantation, exposure to intraoperative heparin did not result in clinical recurrence (Selleng et al. 2008). Even a brief course of heparin should be avoided in patients with a persistently positive functional assay (acute HIT). If surgery cannot be delayed, an alternative anticoagulant such as bivalirudin may be used (Kiser and Fish 2006; Joseph et al. 2014). An alternative approach involves preoperative plasma exchange to remove platelet-activating antibodies and enable intraoperative heparin. This approach appears to be safe, but has not been extensively studied (Warkentin et al. 2015b; Welsby et al. 2010). Whenever a patient with a history of HIT is reexposed to heparin, exposure should be limited to the intra- and early postoperative period (HIT antibodies cannot recur for at least 5 days (Warkentin and Sheppard 2014)). If pre- or postoperative anticoagulation is indicated, a non-heparin anticoagulant should usually be used.

In light of its documented efficacy and safety in large coronary angiography trials (Lehman and Chew 2006), we recommend bivalirudin for heart catheterization including PCI in all patients with a history of HIT. If bivalirudin is not available, heparin is an acceptable alternative in patients with remote HIT, provided that its use is limited to the procedure itself.

Although anti-PF4/heparin antibodies are present in ~10 % of patients on chronic hemodialysis (Carrier et al. 2007; Asmis et al. 2008), HIT is uncommon (<1 %) in this population (Hutchison and Dasgupta 2007). In patients with a history of HIT, treatment with heparin during hemodialysis is contraindicated. Alternative strategies for prevention of dialysis circuit thrombosis, including saline flushing, regional citrate, danaparoid, argatroban, and VKA, have been reported but not systematically investigated.

Take Home Messages

- HIT is one of the most frequent adverse drug effects due to the widespread use of heparin. The clinical features of HIT are summarized in the 4 Ts score, which can be used to determine the clinical likelihood of HIT in the individual patient. Antigen assays such as EIAs have a high negative predictive value and can therefore be used to rule out HIT, while only functional assays differentiate non-platelet-activating antibodies from clinically relevant platelet-activating antibodies. The direct thrombin inhibitors argatroban and bivalirudin, and the factor Xa inhibitors danaparoid and fondaparinux, are established options for treatment of HIT (although approval status differs between jurisdictions). The oral direct anticoagulants are promising options for treatment of HIT, but experience is still anecdotal. The pathogenesis of HIT depends on an immune response towards the endogenous protein PF4, which undergoes a conformational change after binding to polyanions. Likely, HIT is a misdirected antibacterial host defense response. Better understanding of the immunopathology of HIT may provide information on the pathogenesis of other antibody-mediated autoimmune disorders.

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Thrombocytopenia in Pregnancy

Shruti Chaturvedi and Keith R. McCrae

Abstract

Thrombocytopenia is a common hematologic problem in pregnant women. The most common cause of thrombocytopenia is gestational thrombocytopenia, which is usually mild, is not associated with bleeding or neonatal thrombocytopenia, and resolves spontaneously postpartum. Gestational thrombocytopenia, however, may be hard to discern from immune thrombocytopenia (ITP), another cause of isolated thrombocytopenia during pregnancy, although thrombocytopenia due to ITP is often more severe and occurs earlier in pregnancy. Preeclampsia is the most common medical disorder of pregnancy and is associated with a constellation of symptoms including hypertension and proteinuria; thrombocytopenia is generally mild, but reflects the severity of underlying preeclampsia. The syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP) is thought by some to be a variant of preeclampsia as it shares some manifestations but also displays unique ones, particularly liver involvement and microangiopathic hemolytic anemia. HELLP may be difficult to discern from primary thrombotic microangiopathies such as thrombotic thrombocytopenic purpura (TTP) and the atypical hemolytic uremic syndrome (aHUS), which are not unique to pregnancy but occur with increased frequency in this setting. Accurate diagnosis of the cause of thrombocytopenia during pregnancy is important, as treatment varies depending on the etiology. Careful consideration of the timing of onset of thrombocytopenia, and associated hematologic and other manifestations, needs to be considered in the differential diagnosis. Though thrombocytopenic disorders may severely compromise the outcomes of some pregnancies, prompt diagnosis and appropriate therapy often lead to successful pregnancy outcomes.

Introduction

Thrombocytopenia, defined as a platelet count less than 150,000/μL, is the second most common hematologic abnormality in pregnancy after anemia, occurring in up to 6–10 % of pregnancies (Sainio et al. 2000; McCrae 2006; Sullivan and Martin 1995). Platelet counts <100,000/μL are less common and are observed in less than 1 % of pregnancies (Rodeghiero et al. 2009). Although most cases of thrombocytopenia in pregnancy are mild and have no adverse outcome for the mother or fetus, moderate to severe thrombocytopenia may lead to bleeding during delivery, and occasional cases may be related to a life-threatening systemic disorder such as a thrombotic microangiopathy (McCrae 2010). Treatment goals are dynamic—in the first and second trimester the goal is to keep the platelet count in a safe range to prevent spontaneous bleeding, while during delivery, procedural risks and the risk of trauma during parturition necessitate a higher platelet count. Moreover, because therapeutic interventions

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used to treat thrombocytopenic disorders in pregnant women may have toxicities unique to pregnancy, management approaches must be carefully considered.

Disorders That Cause Thrombocytopenia in Pregnancy

There are several potential causes of thrombocytopenia in a pregnant woman. Some of these are specific to pregnancy, others are exacerbated by pregnancy, whereas others may have no relationship to pregnancy at all (Table 1). Gestational thrombocytopenia, hypertensive disorders including preeclampsia, and immune thrombocytopenia (ITP) are the three most frequently encountered causes of thrombocytopenia in the pregnant patient (Table 2) (Burrows and Kelton 1990b; Gernsheimer and McCrae 2007). Thrombotic thrombocytopenic purpura and the HELLP syndrome are rare but must not be missed since they are associated with high maternal and fetal mortality. The evaluation of a pregnant woman presenting with thrombocytopenia is outlined in Table 3.

Table 1 Causes of thrombocytopenia in pregnancy

Pregnancy-specific conditions	Conditions associated with or exacerbated by pregnancy	Nonpregnancy-associated conditions
Gestational thrombocytopenia (70–80 %) Hypertensive disorders (preeclampsia) (15–20 %) HELLP syndrome (<1 %) Acute fatty liver of pregnancy (<1 %)	Thrombotic thrombocytopenic purpura Hemolytic uremic syndrome Disseminated intravascular coagulation (due to HELLP, amniotic fluid embolism, etc.) Type IIB von Willebrand disease-associated thrombocytopenia	Pseudo-thrombocytopenia Immune thrombocytopenia Drug induced thrombocytopenia Congenital thrombocytopenia Hypersplenism Antiphospholipid antibody syndrome Viral infections Nutritional deficiencies (folate, vitamin B12) Bone marrow disorders including aplastic anemia and hematologic malignancies

Table 2 Characteristics of thrombocytopenic disorders in pregnancy

	Gestational thrombocytopenia	Immune thrombocytopenia	Preeclampsia	HELLP syndrome	Thrombotic thrombocytopenic purpura	Atypical HUS
Incidence during pregnancy (%)	5–9	<1	5–8	0.5–0.9	<1	<1
Pathogenesis	Dilutional, increased platelet clearance	Autoimmune	Abnormal placentation	Abnormal placentation, complement regulatory protein mutations in some	Congenital or acquired severe ADAMTS13 deficiency	Complement activation, complement regulatory protein mutations
Timing in pregnancy	Onset late second or third trimester	Any, most common cause in first trimester	Onset in late second or third trimester (>20 weeks gestation)	70 % in late second or third trimester, 30 % postpartum	Second or third trimester	Usually postpartum
Platelet count	Usually >70,000/μL	<100,000/μL, may be severe	Any	Any <100,000/μL	Any <150,000/μL	Any <150,000/μL
Other important laboratory findings	None		24 h urine protein > 0.3 g/24 h	MAHA, LDH > 600 U/L or bilirubin > 1.2 mg/dL, AST > 70 U/L	ADAMTS13 activity <10 %	MAHA, ADAMTS13 not severely reduced
Neonatal thrombocytopenia	No	Yes	No[a]	No[a]	No[a]	No[a]
Resolves postpartum	Yes	May resolve	Yes	Yes	Not without plasma exchange (acquired TTP) or plasma infusion (congenital TTP)	Postpartum onset in most cases

[a]May be associated with thrombocytopenia in the setting of fetal distress and premature delivery

Table 3 Diagnostic evaluation for thrombocytopenia in pregnancy

History
Prior thrombocytopenia or bleeding especially with previous pregnancies, drug intake, autoimmune disorders, systemic illness
Physical examination
Bleeding manifestations, blood pressure (preeclampsia), and abdominal tenderness (HELLP and acute fatty liver of pregnancy)
Laboratory tests
In all patients
Complete blood count
Peripheral blood smear
Reticulocyte count
Liver function tests
Viral screening (hepatitis B virus, hepatitis C virus, human immunodeficiency virus)
When clinically indicated
Lupus anticoagulant and antiphospholipid antibodies
ADAMTS13 activity and inhibitory antibody
Thyroid function testing
Mutations in complement regulatory genes (atypical hemolytic uremic syndrome)
Haptoglobin, lactate dehydrogenase
Direct antiglobulin test (appropriate to rule out Evans syndrome)
Von Willebrand type IIB testing
Disseminated intravascular coagulation testing—prothrombin time, activated partial thromboplastin time, fibrinogen, fibrin split products

Gestational Thrombocytopenia

Gestational thrombocytopenia, also called incidental thrombocytopenia of pregnancy, is the most common cause of thrombocytopenia in pregnant women, accounting for 70–80 % of cases (Burrows and Kelton 1993a; Sainio et al. 2000; McCrae 2006). Normal pregnancy is associated with a physiologic fall in the platelet count that is about 10 % lower than in nonpregnant women (Boehlen et al. 2000; Jensen et al. 2011). It has been speculated that these changes may reflect dilution, decreased platelet production, or accelerated platelet clearance (Provan et al. 2010). The majority of women still have platelet counts within the normal range; however, some women develop counts that fall into the thrombocytopenic range, becoming particularly apparent in the late second or third trimesters. Although there is no established minimum platelet count for gestational thrombocytopenia, most experts agree that this diagnosis is less likely when platelet counts fall to less than 70,000/μL. There are reports of women with more severe thrombocytopenia that did not respond to corticosteroid therapy and resolved postpartum, consistent with gestational thrombocytopenia (Win et al. 2005).

Because there is no diagnostic testing available for gestational thrombocytopenia, it is a diagnosis of exclusion. Patients with a history of primary or secondary ITP, thrombocytopenia preceding pregnancy, an identifiable cause for thrombocytopenia other than pregnancy, or thrombocytopenia that improves with steroid therapy are generally not considered to have gestational thrombocytopenia.

Gestational thrombocytopenia is not associated with adverse maternal or fetal outcomes. The degree of maternal thrombocytopenia is generally not severe enough to increase the risk of bleeding with delivery, although platelet counts less than 75–80,000/μL may compromise the ability to give epidural anesthesia (Provan et al. 2010; van Veen et al. 2010). A brief trial of ITP-directed therapy (corticosteroids or intravenous immunoglobulin) may be considered in patients with platelet counts in the 50,000–70,000/μL range and may have diagnostic and therapeutic utility (McCrae 2010). In the absence of response to steroids, platelet transfusions may be used to raise the platelet count to a "safe" level for epidural catheter placement. Gestational thrombocytopenia is self-limited and resolves 1–2 months after delivery. It may recur in subsequent pregnancies. Importantly, unlike ITP, it is not associated with a higher rate of fetal or neonatal thrombocytopenia compared to non-thrombocytopenic women. When this occurs, it may be due to coincident neonatal alloimmune thrombocytopenia due to maternal exposure and sensitization to fetal platelet antigens (Burrows and Kelton 1988; Curtis 2015).

Immune Thrombocytopenia

ITP is an uncommon cause of thrombocytopenia in pregnancy, occurring in between 1 in 1000 and 1 in 10,000 pregnancies and accounting for about 3 % of pregnant women with isolated thrombocytopenia (Gernsheimer and McCrae 2007). About a third of women with pregnancy-associated ITP are first diagnosed during pregnancy, while two-thirds have a preexisting diagnosis of ITP (Webert et al. 2003). Despite improved understanding of pathophysiology, the factors that cause ITP and may cause worsening during pregnancy are incompletely understood.

The clinical features of ITP in a pregnant woman are similar to those in nonpregnant patients, with bruising, mucosal bleeding, petechiae, and sometimes spontaneous bleeding whose severity usually reflects the degree of thrombocytopenia. ITP can occur at any time during pregnancy; however it is the most common cause of thrombocytopenia in the first and early second trimesters (Gill and Kelton 2000). It is more frequently associated with platelet counts $<$100,000/μL (Provan et al. 2010) and occasionally presents with severe thrombocytopenia and bleeding. Patients with ITP more often have a prior history of ITP or other autoimmune disorders that may be helpful in making this diagnosis.

Management of ITP in Pregnancy

The goal of treatment of ITP in pregnancy is to prevent bleeding. Therefore, treatment is generally not required in the first or second trimester unless the patient has a platelet count less than 20,000–30,000/μL, symptomatic bleeding, or

an elective procedure requiring a higher platelet count. In the third trimester, a higher platelet count is desirable for delivery, especially if the patient desires epidural anesthesia. Recent guidelines recommend a platelet count of at least 75,000/μL for safe placement of an epidural catheter (Provan et al. 2010). It may be advisable to maintain a platelet count of at least 50,000/μL beyond the mid-third trimester in case an emergency cesarean section is required (McCrae 2010). Figure 1 summarizes the management of ITP in pregnancy.

As with nonpregnant patients, corticosteroids are first-line therapy for ITP in pregnancy (Neunert et al. 2011; Provan et al. 2010). We usually start with a low dose of 10–20 mg prednisone daily and titrate to maintain a platelet count >30,000/μL. Prednisolone and prednisone are preferred to dexamethasone, which crosses the placenta more easily. Corticosteroids may have unique toxicities in pregnancy, such as gestational diabetes, pregnancy-induced hypertension, and psychosis. These agents have also been associated with placental abruption, premature rupture of the membranes, and adverse pregnancy outcomes (Kelton 2002; Laskin et al. 1997). Some reports have associated exposure to high-dose corticosteroids in the first trimester with developmental abnormalities such as cleft lip and palate (Carmichael et al. 2007; Czeizel and Rockenbauer 1997; Laskin et al. 1997). Given these potential adverse effects, steroids should be used sparingly and at the lowest possible dose. Corticosteroids should be tapered, with close monitoring in the postpartum period to avoid a rapid fall in the platelet count.

For patients who respond suboptimally, or not at all, to reasonably tolerated doses of corticosteroids, intravenous immunoglobulin (IVIg) at a dose of 1 g/kg (in single or divided doses) can be used (Provan et al. 2010). Indeed, some have cogently argued that due to the toxicity of corticosteroids, IVIg should be used as the first-line therapy for pregnancy-associated ITP. The duration of response to IVIg is short, lasting 2–3 weeks, and repeated infusions are usually required to maintain adequate platelet counts. For patients who do not respond to corticosteroids or IVIg as single agents, combinations of these therapies may be effective, particularly when higher-dose "pulse" steroids are used (e.g., methylprednisolone 1 g/kg for 2 consecutive days). This approach, along with platelet transfusions, may be needed close to delivery (Koyama et al. 2012; Webert et al. 2003).

Occasionally, a patient may be refractory to treatment with corticosteroids and IVIg. For these refractory patients, toxicity and teratogenicity of alternative agents limit their use. Splenectomy, although rarely necessary, may safely be performed laparoscopically in the mid-second trimester to avoid early pregnancy loss or obstruction of the surgical field by the gravid uterus closer to term (Griffiths et al. 2005). The recommended pre-splenectomy vaccines (meningococcal, pneumococcal, and *Haemophilus influenzae* B vaccines) are all inactivated and can be safely administered during pregnancy (Keller-Stanislawski et al. 2014; Chu and Englund 2014).

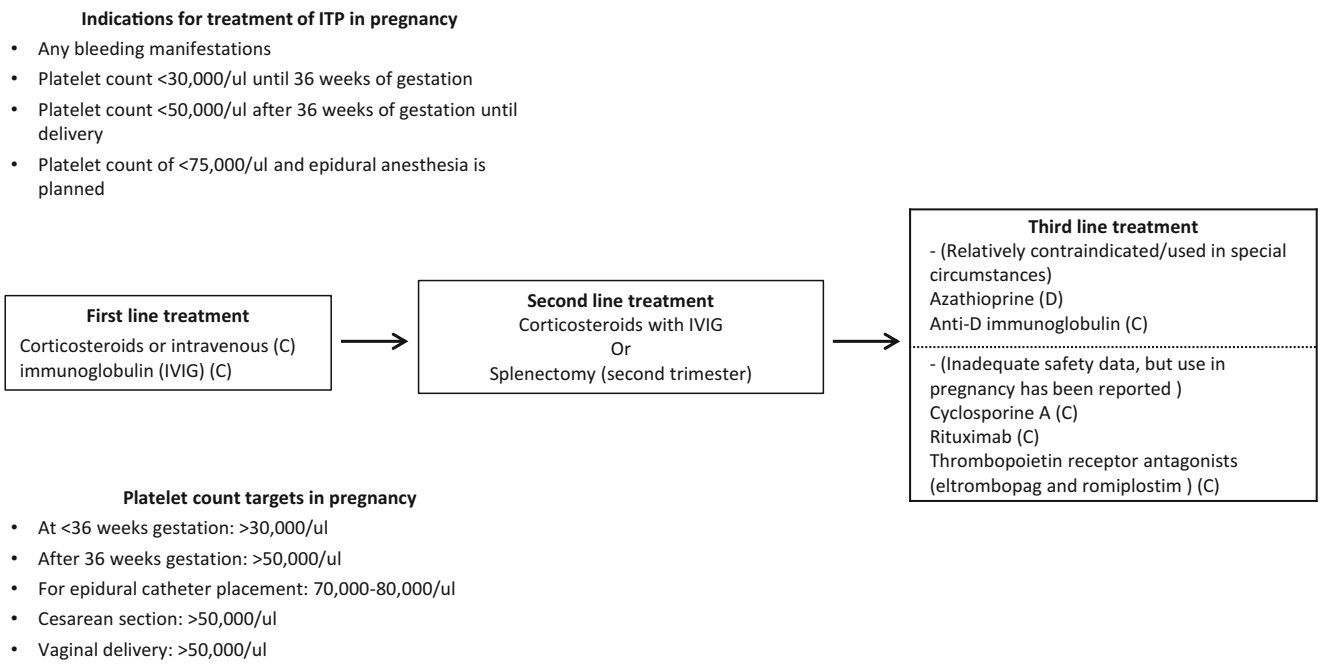

Fig. 1 Management of ITP in pregnancy. *Letters in brackets* indicate FDA-designated pregnancy category. (C) = studies in animals show risk, but inadequate studies in human fetuses. Benefit may justify risk; (D) = evidence of risk in human fetuses. Benefit may justify risk; (X) = studies in animals or human fetuses demonstrate abnormalities. Risk of harm outweighs benefits

Azathioprine has been used safely in pregnant women with renal transplants, systemic lupus erythematosus, and inflammatory bowel disease (Erkman and Blythe 1972; Price et al. 1976), suggesting that it is reasonably safe for use in pregnancy-associated ITP. Some studies report associations of maternal azathioprine with intrauterine growth restriction, preterm delivery, and immune impairment of the neonate (Sukenik-Halevy et al. 2008; Gisbert 2010). It is labeled as pregnancy category D by the US Food and Drug Administration (FDA), indicating that there is positive evidence of fetal risk, but benefits may outweigh the risk in case of serious or life-threatening disease. Its delayed effect, however, limits its use as a steroid-sparing agent. Cyclosporin A has also been used safely in pregnancy. In general, cytotoxic agents are associated with a risk of teratogenicity and should be avoided if possible.

There is limited data regarding the use of rituximab in pregnancy. There are several reports of its successful use in pregnancy-associated ITP (Gall et al. 2010; Klink et al. 2008). Rituximab crosses the placenta and may cause a delay in neonatal B-cell maturation. This resolves by 4–6 months, and reports to date have not showed any clinically significant infections in the offspring (Klink et al. 2008; Gall et al. 2010). One retrospective study identified 153 pregnancies associated with rituximab exposure for various indications, with known outcomes (Chakravarty et al. 2011). There were 90 live births, of which 22 were preterm. Eleven neonates had hematologic abnormalities (lymphopenia, neutropenia, or B-cell depletion) that were not associated with infectious complications (Chakravarty et al. 2011). Rituximab has been designated pregnancy category C by the FDA (Chakravarty et al. 2011).

There are isolated case reports of the use of thrombopoietin receptor antagonists (eltrombopag and romiplostim) for refractory ITP in pregnancy (Alkaabi et al. 2012; Decroocq et al. 2014); however their effects on the fetus are unknown, and their use cannot be recommended in pregnancy. Short-term therapy with danazol in combination with high-dose IVIg and corticosteroids has been used for refractory thrombocytopenia in the third trimester (Orisaka et al. 2005). However, danazol has been observed to cause birth defects and has been designated category X by the FDA; its use should therefore be avoided.

Risk of Neonatal Thrombocytopenia

Anti-platelet antibodies can cross the placenta and cause fetal and neonatal thrombocytopenia. The development and severity of neonatal thrombocytopenia depend on many poorly understood factors including the stage of pregnancy (greatest in the third trimester), maturity of the fetal reticuloendothelial system, and immunoglobulin subclass (highest for IgG1) (Kane and Acquah 2009). There is no consistent correlation between neonatal thrombocytopenia and the severity of maternal thrombocytopenia, the level of maternal platelet-associated immunoglobulin, whether or not the mother has undergone splenectomy, or several other parameters (Kelton 2002; Stavrou and McCrae 2009; Burrows and Kelton 1993b). In fact, the best predictor of neonatal thrombocytopenia is a history of thrombocytopenia in an older sibling (Christiaens et al. 1997).

Thrombocytopenia, with a platelet count <50,000/μL, occurs in approximately 10 % of newborns of mothers with ITP, whereas platelet counts <20,000/μL occur in approximately 4 % (Burrows and Kelton 1993b). There is no safe and reliable indirect method to measure the fetal platelet count. Neonates with severe thrombocytopenia may experience bleeding complications, the most feared of which is intracranial hemorrhage. Because of a perceived increased risk of intracranial hemorrhage as a consequence of head trauma during vaginal delivery, in previous decades nearly all pregnant patients with ITP were managed with cesarean section. However, more recent data have shown that (a) the risk of fetal intracranial hemorrhage in offspring of mothers with ITP is extremely low (Burrows and Kelton 1993b), and (b) this risk is not substantially increased by vaginal delivery (Cook et al. 1991). Therefore, maternal ITP is not an indication for cesarean delivery, and the delivery method should be guided by maternal indications only. Antenatal determination of the fetal platelet count by percutaneous umbilical cord blood sampling (or cordocentesis) is not recommended since it is associated with a 1–2 % risk of fetal loss, at least as high as the risk of intracranial hemorrhage due to maternal ITP (Stavrou and McCrae 2009). It must be noted that low-dose maternal corticosteroids do not increase neonatal platelet count and should not be used with this objective (Christiaens et al. 1990).

The neonatal platelet count should be measured on a cord blood or peripheral blood sample immediately after delivery. If completely normal, this does not need to be rechecked, but parents should be counseled to watch for bruising petechiae or signs of bleeding. For neonates with thrombocytopenia, platelet counts should be checked regularly for the first week, since the platelet nadir is frequently observed at 2–5 days of life (Burrows and Kelton 1990a). Neonates with platelet counts <50,000/μL should undergo a transcranial ultrasound to rule out intracranial hemorrhage, even if asymptomatic. Platelet transfusions and IVIg may be used to treat neonates with platelet counts <30,000/μL, or those with active bleeding (Gibson et al. 2004).

Thrombotic Thrombocytopenic Purpura and Other Thrombotic Microangiopathies

Thrombotic Thrombocytopenic Purpura (TTP)

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening systemic disorder characterized by microangiopathic hemolytic anemia (MAHA), thrombocytopenia, and varying degrees of renal, neurologic, and other organ

dysfunctions (Kremer Hovinga 2017 in this volume). It is caused by a deficiency of ADAMTS13, a von Willebrand factor-cleaving protease. Deficiency of ADAMTS13 is usually acquired, resulting from neutralizing autoantibodies (Tsai and Lian 1998), although congenital ADAMTS13 deficiency (Upshaw-Schulman syndrome) accounts for a minority of cases, many of which may present for the first time during pregnancy (Lotta et al. 2010). Although TTP is not pregnancy specific, it occurs with increased frequency during pregnancy, predominantly in the second and third trimester (Scully et al. 2014; Martin et al. 2008; Kremer Hovinga et al. 2010). Although the pathophysiology underlying this association is not well understood, it may be related to the increases in fibrinogen, factor VIII, VWF, and factor VIIa (Stirling et al. 1984) and the decrease in ADAMTS13 activity that occurs during normal pregnancy. In one study, the mean ADAMTS13 activity decreased from 94 % (range 40–160 %) in the first trimester to 64 % (range 22–135 %) in the second and third trimesters (Mannucci et al. 2001); however these levels are well above the 5–10 % levels associated with TTP.

TTP is a challenging diagnosis to make in pregnancy, since its clinical features overlap with other pregnancy-specific conditions such as preeclampsia, HELLP syndrome, and acute fatty liver of pregnancy (AFLP) that often manifest with MAHA and thrombocytopenia. Measuring ADAMTS13 activity and inhibitory antibody helps in confirming the diagnosis; however these tests may not be immediately available. Due to the importance of prompt initiation of therapy for this disorder, any patient with unexplained MAHA and thrombocytopenia should be considered to have TTP and started on daily plasma exchange, which has a response rate of approximately 80 %. The role of corticosteroids in pregnancy-associated TTP has not been evaluated in randomized trials. Although they are commonly used in conjunction with plasma exchange in the nonpregnant patient with TTP, there may be an increased risk of complications in pregnant individuals. TTP can cause thrombosis in the placental circulation and result in fetal growth restriction, intrauterine fetal death, and preeclampsia. Therefore all pregnant women with a history of TTP should be followed by a high-risk obstetrician and undergo regular fetal growth scans and uterine artery Doppler monitoring. The British Committee for Standards in Hematology guidelines also advise thromboprophylaxis with aspirin and low molecular weight heparin in a patient treated for TTP once the platelet count reaches 50,000/μL (Scully et al. 2012).

Women with Upshaw-Schulman syndrome (congenital ADAMTS13 deficiency) frequently have acute episodes of TTP during pregnancy, and these are associated with high fetal mortality. Indeed, patients with congenital TTP are disproportionately represented in pregnancy-associated TTP (Moatti-Cohen et al. 2012; Scully et al. 2014). It is important to distinguish congenital from acquired TTP, as regular plasma infusions may prevent acute attacks and permit a normal pregnancy outcome in congenital TTP, while acquired TTP should be treated with daily plasma exchange for days or even weeks to induce remission (Scully et al. 2014; von Auer et al. 2015). It is important to note that, unlike preeclampsia, eclampsia, and HELLP syndrome, delivery does not lead to resolution of TTP.

There is an increased risk for TTP in subsequent pregnancies; however these pregnancies can be successfully managed with close monitoring and care. One series reported successful outcomes in 18 pregnancies in women with a history of congenital TTP who were managed with prophylactic plasma infusions and close monitoring (Scully et al. 2014). In another series of 10 women with acquired TTP, recurrent TTP occurred in 2 of 16 pregnancies, while 13 (81 %) pregnancies resulted in normal births (Jiang et al. 2014). Patients with a history of TTP may be at higher risk of preeclampsia.

Hemolytic Uremic Syndrome

Hemolytic uremic syndrome (HUS) is a thrombotic microangiopathy that is pathophysiologically distinct from TTP, but shares overlapping clinical features. HUS is typically associated with the presence of acute renal failure, which is less commonly observed in TTP (Coppo et al. 2006) (Kremer Hovinga 2017 in this volume). Levels of ADAMTS13 are generally not severely reduced in most patients with HUS. Prior to the availability of ADAMTS13 testing, it was nearly impossible to definitively distinguish HUS from TTP. While TTP/HUS is estimated to occur in 1:25,000 pregnancies (Dashe et al. 1998), contemporary estimates of the incidence of HUS per se during pregnancy are not available.

There are several variants of HUS. One type, D+ HUS, is caused by infection with Shiga toxin-producing *E. coli* (typically types O157:H7 and O104:84). However, the commonest type of HUS encountered during pregnancy is atypical HUS (aHUS), which is associated with activation of the alternate complement pathway (Fakhouri et al. 2010; Nester and Thomas 2012). Patients with atypical HUS may have mutations in complement regulatory proteins such as factor H, factor I, membrane cofactor protein, or thrombomodulin, among others, as well as activating mutations in complement factors B and C3. Factor H-reactive antibodies may cause acquired factor H deficiency. In a retrospective cohort of 100 women with atypical HUS, 21 % had pregnancy-associated atypical HUS of which 79 % presented in the postpartum period (Fakhouri et al. 2010). Complement abnormalities were detected in 18 of the 21 patients with pregnancy-associated atypical HUS. Overall outcomes were poor with 4.8 % and 7.7 % associated with fetal loss or preeclampsia, respectively. Seventy six percent of patients developed end-stage renal disease despite plasma exchange. However, this data was accumulated prior to the availability of eculizumab, a

humanized monoclonal anti-C5 antibody that is highly effective in treating atypical HUS.

Preeclampsia and the HELLP Syndrome

These disorders are discussed together, because they share similar clinical characteristics and pathophysiology. Preeclampsia affects up to 6 % of all first pregnancies (American College of Obstetricians and Gynecologists [ACOG] practice bulletin 2002), accounts for 21 % cases of thrombocytopenia at delivery, and is the most common cause of pregnancy-associated mortality worldwide. ACOG criteria for the diagnosis of preeclampsia include the following: (1) blood pressure of 140 mmHg systolic or 90 mmHg diastolic or higher that occurs after 20 weeks of gestation in a woman with previously normal blood pressure, and (2) proteinuria, defined as urinary excretion of 0.3 g of protein or higher in a 24-h specimen that usually correlates with a 1+ or greater reading on dipstick (ACOG practice bulletin 2002). Multiple organ systems are affected in preeclampsia, reflecting systemic endothelial dysfunction, although the kidneys are affected most severely. Predisposing factors include age <20 years or >30 years, a high body mass index, chronic hypertension, as well as maternal and paternal genetic factors (McCrae 2010; Mostello et al. 2008). Thrombocytopenia is observed in about 50 % of patients with preeclampsia and generally parallels the severity of preeclampsia.

The HELLP syndrome develops in 10–20 % of women with severe preeclampsia and affects 0.5–0.9 % of all pregnancies (Haram et al. 2009; Kirkpatrick 2010). Criteria for the diagnosis of HELLP syndrome include hemolysis (abnormal peripheral smear, LDH > 600 U/L, or bilirubin > 1.2 mg/dL), aspartate aminotransferase > 70 U/L, and a platelet count < 100×10^9/L (Sibai 2011). The risk of severe complications in the HELLP syndrome parallels the severity of thrombocytopenia, although severe complications such as hepatic rupture and hemorrhage can occur even without severe thrombocytopenia.

Abnormal placentation, the process by which fetal trophoblast cells invade the maternal decidua and remodel the maternal uterine spiral arteries, is central to the pathogenesis of preeclampsia, in which both the depth of trophoblast invasion and the extent of remodeling of the spiral arteries are reduced. This may reflect deficiencies in trophoblast function, including failure of trophoblast cells to alter their pattern of integrin expression toward an endothelial phenotype and deficient protease activity, among others (Young et al. 2010). As pregnancy advances, insufficient placentation results in progressive uteroplacental ischemia that induces the systemic manifestations of preeclampsia. As early as the first trimester, patients who are destined to develop preeclampsia exhibit elevated levels of soluble vascular endothelial cell growth factor (VEGF) receptor type 1 (sFlt1) (Maynard et al. 2005), as well as endoglin, an endothelial cell-derived member of the tumor growth factor-β (TGF-β) receptor family (Venkatesha et al. 2006). Increased levels of sFlt1 and endoglin mRNA are present in preeclamptic placentae, suggesting this is the source of these proteins. sFlt1 binds and neutralizes VEGF and placental growth factor (PLGF), and endoglin blocks the binding of TGF-β to endothelial cells. One outcome of these actions is to decrease expression of endothelial nitric oxide (NO) synthase leading to reduced NO production and exacerbation of the hypertensive manifestations of preeclampsia. Functional deficiency of VEGF/PLGF also results in endothelial dysfunction particularly that of the glomerular endothelium, leading to the characteristic endothelial swelling and "glomerular endotheliosis" lesions of preeclampsia and in some cases the development of a thrombotic microangiopathy (Myatt and Webster 2009). Mutations in genes regulating the activity of the alternative complement system (factor H, factor I, and membrane cofactor protein) were detected in 4 of 11 patients with HELLP syndrome and renal involvement (Fakhouri et al. 2008), suggesting that, as in atypical hemolytic uremic syndrome (aHUS), excessive complement activation may be involved in the pathogenesis of HELLP in some patients.

The mainstay of treatment for preeclampsia and HELLP syndrome is delivery of the fetus. Prompt delivery is indicated in women beyond 34 weeks of gestation, or with fetal distress or severe maternal disease (Lindheimer et al. 2010). For pregnancies <34 weeks of gestation and in which the maternal and fetal status is stable, delivery may be attempted after administering beclomethasone to accelerate fetal lung maturity (Roberts and Dalziel 2006). In addition to medical stabilization and prompt delivery, most patients require additional antihypertensive medications and should receive magnesium sulfate for seizure prophylaxis. The use of high-dose corticosteroids and plasma exchange remain controversial, but some series indicate that these may improve the outcomes of severe HELLP (Woudstra et al. 2010; Eser et al. 2005).

Women with preeclampsia or HELLP are at increased risk for development of recurrent disease and poor pregnancy outcome in subsequent pregnancies (Dildy et al. 2007). A meta-analysis suggests that aspirin has modest efficacy in prevention of preeclampsia, although no difference in the incidence of fetal death was demonstrated (Askie et al. 2007). In recent years, it has become increasingly apparent that patients with preeclampsia are at increased risk for cardiovascular disease and death during long-term follow-up (Cirillo and Cohn 2015).

Acute Fatty Liver of Pregnancy

Acute fatty liver of pregnancy is a rare disorder that usually presents in the third trimester of pregnancy with nausea, vomiting, malaise, right upper quadrant pain, and cholestatic

liver dysfunction (Fesenmeier et al. 2005). It occurs in 1:7000–1:20,000 deliveries and has a maternal mortality rate of around 15 %. Approximately 50 % patients with AFLP meet the criteria for preeclampsia, but overlap with HELLP may also occur. Most patients develop DIC due to acquired antithrombin deficiency, with thrombocytopenia and deficiencies of fibrinogen and other clotting factors (Castro et al. 1999). Due to the coagulopathy, bleeding is common, despite only mild thrombocytopenia. Other complications include hypoglycemia, infection, renal insufficiency, and encephalopathy. Some cases of acute fatty liver, as well as HELLP, may be associated with fetal mitochondrial fatty acid oxidation defects, most commonly due to deficiency of long-chain 3-hydroxyacyl-coenzyme A dehydrogenase. Treatment involves supportive care with blood product support for the underlying coagulopathy (McCrae 2006). Delivery of the fetus leads to improvement in AFLP within 2–3 days and should be seriously considered in pregnancies beyond the period of viability, fetal distress, or life-threatening maternal disease. Plasma exchange, with or without continuous hemofiltration, has also been reported to improve outcomes in severe AFLP (Jin et al. 2012; Yu et al. 2014).

Miscellaneous Causes of Thrombocytopenia

In this section, we will briefly discuss several miscellaneous causes of thrombocytopenia in pregnancy. These, and other causes that are not discussed, are listed in Table 1.

In all individuals with thrombocytopenia, whether pregnant or non-pregnant, the peripheral blood film should be examined to evaluate for ethylenediaminetetraacetic acid (EDTA)-dependent platelet clumping, causing "pseudothrombocytopenia." In such cases, determination of the platelet count in a tube containing a non-EDTA anticoagulant such as citrate-tris-pyridoxalphosphate (CPT) may eliminate clumping and lead to more accurate readings (Podda et al. 2012).

Disseminated intravascular coagulation (DIC) may arise from a number of events in pregnant women. Placental abruption, amniotic fluid embolism, and uterine rupture cause fulminant DIC due to the rapid release of tissue factor-rich material that causes profound activation of coagulation, with consumption of coagulation factors and severe hypofibrinogenemia. DIC may, however, develop more gradually in the case of retained fetal products, and thrombocytopenia may be the presenting feature (McCrae and Cines 1997).

The antiphospholipid syndrome may be associated with immune thrombocytopenia or a microangiopathic thrombocytopenia (Asherson et al. 2007). Treatment strategies similar to primary ITP, with corticosteroids and IVIg, may be employed when platelet count is <30,000/μL, with azathioprine, rituximab, or other interventions reserved for refractory cases (Gernsheimer and McCrae 2007). Treatment of thrombocytopenia in these patients must be balanced with the risk of thrombosis and pregnancy loss. In women with a persistent lupus anticoagulant or antiphospholipid antibodies and no prior history of thrombosis, daily low-dose aspirin may be started if the platelet count is greater than approximately 50,000/μL. If a history of prior spontaneous abortion or thrombosis is present, the patient should be treated with aspirin and low molecular weight heparin. A stable platelet count >50,000/μL is usually considered safe for anticoagulation (Gernsheimer and McCrae 2007).

Type IIB von Willebrand disease is characterized by a mutant von Willebrand factor (VWF) molecule that binds to its primary platelet receptor, glycoprotein Ib, with increased affinity, thereby inducing platelet agglutination, accelerated platelet clearance, and thrombocytopenia (Grainick et al. 1985). Levels of endogenous VWF increase during pregnancy (Stirling et al. 1984), and thrombocytopenia induced by the mutant VWF may become more apparent (Rick et al. 1987). In some patients, platelet counts can fall to as low as 20,000–30,000/μL, with improvement after delivery. Desmopressin can worsen thrombocytopenia and should not be used. Platelet transfusions along with a VWF-containing factor VIII concentrate have been used to maintain safe platelet counts and prevent hemorrhage during delivery (Hepner and Tsen 2004; Ieko et al. 1990). Phenotypically similar to type IIB von Willebrand disease is platelet-type von Willebrand disease or pseudo-von Willebrand disease. This is an autosomal dominant disorder characterized by gain of function mutations of the glycoprotein Iba (GP1BA) gene, resulting in enhanced binding of VWF to the platelet surface receptor, and accelerated platelet clearance. Distinguishing the two conditions may be difficult but has important clinical implications; patients with platelet-type von Willebrand disease need to be treated with platelet transfusions rather than VWF replacement because of their inherent platelet defect and thrombocytopenia. The commonly used ristocetin-induced platelet aggregation is unreliable in view of the physiological increases in platelet activation and adhesion in pregnancy (Ranger et al. 2012); however, platelet aggregation studies in the presence of normal plasma VWF from cryoprecipitate have been successfully used to make this diagnosis (Enayat et al. 2006). Confirmatory diagnosis can also be made by genotypic analysis.

Drug-induced thrombocytopenia may result from a variety of prescription drugs, as well as illicit medications, such as cocaine. While *infiltrative marrow disorders* (e.g., hematologic malignancies, metastatic cancer, invasive fungal or other infections) and bone marrow failure syndromes (e.g., aplastic anemia, myelodysplasic syndrome, and

myelofibrosis) can cause cytopenias, including thrombocytopenia, these are uncommon in women of childbearing age. *Severe nutritional deficiencies*, such as folate or vitamin B12 deficiencies, can present with thrombocytopenia, usually along with other cytopenias, but these are similarly rare in pregnant women. *Congenital thrombocytopenias*, such as the May-Hegglin anomaly, may first be detected during pregnancy and may be detected through identification of abnormal platelet morphology on review of the peripheral blood film of the patient and family members.

Take-Home Messages

- Gestational thrombocytopenia, hypertensive disorders of pregnancy, and immune thrombocytopenia are the most common causes of low platelet counts in a pregnant woman.
- Time of onset during pregnancy, careful clinical and laboratory evaluation, and attention to associated clinical findings need to be considered to make an accurate diagnosis of the cause of thrombocytopenia in a pregnant woman.
- Treatment depends on etiology and is aimed at preventing hemorrhage—platelet count targets depend upon the period of gestation and planned procedures.
- HELLP syndrome and TTP/HUS are life-threatening diagnoses that must be excluded in all patients.

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Thrombocytopenia in the Newborn

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Abstract

Thrombocytopenia is common in preterm and sick newborn infants. Establishing the cause and deciding at what threshold to transfuse donor platelets is a recurrent clinical challenge. A healthy baby will have a platelet count that is in the *normal range* ($>150 \times 10^9$/L) at birth, but there is evidence that the progenitor pool is limited and thrombopoiesis vulnerable to disruption and exhaustion, particularly at very early gestational ages.

This chapter gives an overview of current understanding of the *physiology of platelet production and function* in the newborn and describes the aetiology and natural history of conditions that lead to *thrombocytopenia in clinical practice*. The relationship between *thrombocytopenia and clinical bleeding* remains poorly defined, with appropriate thresholds for platelet transfusion subject to debate. Current UK *transfusion guidelines* are presented, along with ongoing studies, to establish an evidence base for what remains often pragmatic practice. This chapter ends with a look towards therapeutic *alternatives to transfusion* of donor platelets for prevention and treatment of thrombocytopenia in the newborn.

Introduction

Thrombocytopenia is common in neonates admitted to critical care, particularly in low gestational age babies. As this group of patients are at high risk of clinically significant and potentially damaging bleeding (Wilson-Costello et al. 2007; Stoll et al. 2015), prophylactic transfusion of platelets is common in neonatal practice. However, predictors of clinically important bleeding remain uncertain, and hence there is a lack of international consensus regarding appropriate thresholds for triggering platelet transfusion, a therapy that brings its own dangers (Stainsby et al. 2008; Roberts et al. 2008; Christensen 2011).

Normal Ranges

The definition of thrombocytopenia in the newborn is controversial. Most commonly the 'normal' range for platelet count is considered to be the same as for children and adults ($150–450 \times 10^9$/L) so that babies with platelet counts below 150×10^9/L would be considered to be thrombocytopenic, regardless of their gestational age at birth. The basis for this are studies of fetal blood obtained by cordocentesis, which have shown that the mean platelet count has reached 150×10^9/L by the end of the first trimester and rises to between 175 and 250×10^9/L by the end of the second trimester (Holmberg et al. 1983; Forestier et al. 1986,

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1991). This definition is further supported by studies of platelet counts in babies born at term that show that over 98 % have values above 150 × 10^9/L at birth (Burrows and Kelton 1990a, 1993; Sainio et al. 2000).

An alternative view is that definitions should be based on reference ranges from representative patients (Christensen 2011). Wiedmeier and colleagues (2009) reported reference ranges in 2008, based on the 5th to 95th centiles for platelet counts from a large cohort of almost 50,000 neonates born between 22 and 42 weeks gestation, measured in the first 90 postnatal days. They found that platelet counts were generally lower the more preterm the baby is, with a 5th centile of 104 × 10^9/L in babies <32 weeks gestation, whereas in babies >32 weeks it was 123 × 10^9/L. They suggested that platelet counts of 100–150 × 10^9/L should be considered normal in the most preterm infants. Other studies similarly suggest a relationship between platelet count and gestational age (Aballi et al. 1968; Sell and Corrigan 1973; Van den Hof and Nicolaides 1990; Haque and Bahakim 1991; Obladen et al. 2000), although results have not been consistent.

A caution to ascribing these data to a 'normal' range is that they are based on babies in neonatal units, who by virtue of their circumstances would generally not have normal haematological values. If they are premature, they will usually have pathological reasons for their premature birth, most of which are known to have an impact on blood values, such as infection, bleeding or placental problems. Following birth they will often be clinically unstable and subject to significant physiological stresses which, in turn, would be expected to affect their haematological indices. None the less, these studies provide an important reference to guide clinical practice.

Overall there remains reasonable international consensus that thrombocytopenia in the neonate should be defined as a platelet count of less than 150 × 10^9/L. This can be further broken down into mild when the platelet count is 100–150 × 10^9/L, moderate 50–100 × 10^9/L and severe when <50 × 10^9/L.

Following the birth of a healthy full-term baby, there is frequently a rise in platelet count over the first 2–3 postnatal weeks (Christensen 2011; Weidmeier et al. 2009; Aballi et al. 1968; Obladen et al. 2000). Earlier studies suggest that after this initial increase, the platelet count reaches a plateau (Aballi et al. 1968; Sasanakul et al. 1993; Appleyard and Brinton 1971). Weidmeier et al. (2009) in their large cohort study of unselected term and preterm infants showed a sinusoidal pattern of platelet counts after birth and during the first 90 postnatal days, with an initial peak at 2–3 weeks, followed by a relative fall and a secondary peak at 6–7 weeks. However, as the study was based on infants who had blood counts as part of a clinical inpatient or outpatient visit, the sinusoidal pattern might not represent true 'normality'. Weidemeier described the 95th centile in their cohort of babies to increase to over 700 × 10^9/L at 5–6 weeks postnatal age and suggest that in many healthy babies platelet counts rise to values generally considered to indicate thrombocytosis.

Prevalence of Thrombocytopenia

Thrombocytopenia, defined as a platelet count less than 150 × 10^9/L, is common in babies subjected to blood counts in the newborn period. The prevalence varies depending on the population studied, being more frequent in babies with significant illness and especially with lower gestation at birth. Thrombocytopenia has been reported in 1–2 % of all newborns (Dreyfus et al. 1997), rising to 18–35 % of sick babies undergoing intensive care (Roberts et al. 2008; Christensen et al. 2006; Castle et al. 1986; Metha et al. 1980). Preterm babies are particularly likely to develop thrombocytopenia. In a survey of 284 extremely low birth weight (<1000 g at birth) babies in four neonatal intensive care units across the USA, Christensen and colleagues (2006) described platelet counts below 150 × 10^9/L in 73 % of all babies and in up to 90 % of the very smallest babies. In this study, 38 % of babies had platelet counts <50 × 10^9/L. In a large multicentre study in the UK, 5 % of all babies admitted to participating neonatal units, irrespective of birth weight, developed severe thrombocytopenia, defined in this study as <60 × 10^9/L (Stanworth et al. 2009).

Platelet Physiology

Megakaryocytopoiesis in Fetus and Newborn

The study of fetal megakaryocytopoiesis is hampered by the very low numbers of megakaryocytes in fetal bone marrow and the difficulty of obtaining specimens for isolating megakaryocyte progenitors. Available evidence suggests that megakaryocytopoiesis follows a similar pattern developmentally to other haemopoietic lineages, but appears to develop later than erythropoiesis and granulopoiesis. Primitive haemopoiesis has been identified initially in the embryonic yolk sac as early as 19 days from conception, in the embryonic liver by 5–6 weeks and in the bone marrow from 11 weeks. Megakaryocytes have been demonstrated in the yolk sac by 5 weeks and in the liver and spleen by 10 weeks gestation. Megakaryocyte progenitors have been isolated from fetal blood and bone marrow from 18 weeks gestation confirming that megakaryocytopoiesis is well established by the third trimester (Graeve and De Alarcon 1989; Peault et al. 1999; Zon 1995; Huyhn et al. 1995; Zauli et al. 1993; Bruno et al. 1996).

Haemopoiesis occurs predominantly in the liver for much of the second trimester in the human fetus, but declines in the liver to negligible levels by term. Bone marrow haemopoiesis increases over the second trimester, and by 24 weeks the composition of the fetal marrow begins to resemble that of adults. By term almost all haemopoiesis is in the marrow (Peault et al. 1999; Charbord et al. 1996). It is suggested that the changes in sites of haemopoiesis during fetal life follow migration of stem and progenitor cells between tissues via the circulation. This is supported by the finding of large numbers of these cells, including MK-lineage progenitors, in the fetal circulation, which diminish with increasing gestation (Linch et al. 1982; Murray and Roberts 1995). This transitioning of the site of megakaryocytopoiesis may explain some of the vulnerability of newborns, particularly at very early gestations, to disruption of platelet production and development of thrombocytopenia.

Good evidence exists for significant differences between fetal and neonatal megakaryocytopoiesis and that found in adults (Ferrer-Marin et al. 2010). Fetal megakaryocyte progenitors appear to have greater proliferative potential than adult progenitors, producing significantly larger megakaryocyte colonies in vitro cell culture (Murray et al. 1998; Nishihira et al. 1996). In addition, the high proliferative potential cell-megakaryocyte (HPPC-MK) progenitor cell, which expresses a surface marker phenotype similar to primitive human fetal stem cells, has only been isolated from humans using in vitro clonal assays from fetal, and not adult, bone marrow (Bruno et al. 1996).

The relative numbers, metabolism and morphology of megakaryocyte lineage cells also change during fetal development and differ from adults. There is a higher proportion of immature megakaryocyte progenitors in the fetus than in term babies and in adults, and these progenitors also appear to have a higher proliferative capacity and to be more sensitive to cytokine stimulation (Zauli et al. 1993; Bruno and Hoffman 1998; Bruno et al. 1996). However, fetal and neonatal megakaryocytes are significantly smaller and of lower ploidy than adult megakaryocytes, which has been shown to be associated with the production of fewer platelets per progenitor. Fetal megakaryocytes increase in size and ploidy with increasing gestation (Graeve and De Alarcon 1989; Hegyi et al. 1991; de Alarcon and Graeve 1996).

As in adults, thrombopoietin (Tpo) is the predominant cytokine regulating megakaryocytopoiesis and platelet production in the fetus and newborn. However, there appear to be distinct developmental aspects of Tpo metabolism and its response to thrombocytopenia that may be important. Baseline Tpo levels in the immediate newborn period appear to be relatively higher than in adults (Murray et al. 1998; Walka et al. 1999). Studies attempting to elucidate the response of Tpo blood levels to thrombocytopenia in the newborn have yielded conflicting results, with some suggesting an attenuated response, with relatively low Tpo levels in the face of thrombocytopenia secondary to reduced megakaryocytopoiesis (Watts et al. 1999; Sola et al. 1999), whereas other studies have shown that newborn babies can produce high circulating Tpo levels (Colarizi et al. 1999; Sola et al. 2004).

Despite this lack of clarity about Tpo production in the newborn, there is clearer evidence regarding the response of megakaryocyte lineage cells to Tpo and other cytokine stimulation. In vitro data shows that progenitor cells in the fetus and newborn are more sensitive to Tpo stimulation than adult cells, with a greater proliferative response (Nishihira et al. 1996; Murray 1999; Sola et al. 2000). However, megakaryocytes appear to show an attenuated increase in size and ploidy, when subjected to Tpo (Pastos et al. 2006). This further supports the idea that neonates respond to thrombocytopenic stress by increasing progenitor proliferation, rather than enhancing megakaryocyte maturation and platelet production at the distal end of the megakaryopoietic pathway.

If, as these data suggest, the preterm newborn infant must rely on recruitment of additional megakaryocyte progenitors to increase platelet production in times of stress, animal studies aimed at elucidating neonatal granulopoiesis may provide explanation why maintaining normal number of both neutrophils and platelets so often fail. Quantification of fetal progenitors in newborn rodents showed significant reduction in total progenitors relative to adults (Christensen and Rothstein 1984). It has also been shown that in steady state, a higher proportion of human fetal progenitors are actively proliferating at any time, and hence there are fewer to recruit into production in the face of increased utilisation of neutrophils, particularly in response to sepsis (Christensen et al. 1986; Christensen 1988). These data have been used to explain the high frequency of neutropenia in response to neonatal sepsis. It may be surmised that the same principles may apply for platelet production and explain the relatively high incidence of thrombocytopenia in the sick neonate.

The implications of the many differences between adult and newborn megakaryocytopoiesis for the maintenance of adequate platelet numbers in the preterm and sick infant need further study. However, current evidence provides developmental reasons why thrombocytopenia is so frequent and suggests that any biological intervention to increase platelet numbers will need a prophylactic strategy based on risk, rather than a reactive strategy introduced once thrombocytopenia has developed.

Newborn Platelet Function and Primary Haemostasis

The haemostatic system in neonates is similarly distinct from that of adults. Many of the protein components of

secondary haemostasis are at significantly lower concentrations compared to adult values in the first 6 months, and normal ranges for coagulation factors have been established according to gestational age (Kuhle et al. 2003). An understanding of 'immaturity' of platelet function and primary haemostasis might inform practice and justify the use of higher transfusion thresholds for prophylactic platelets in newborns. However, neonatal platelet function and primary haemostasis has been difficult to characterise (Haley et al. 2014). This partially reflects the complexity of primary haemostasis, which is usually conceptualised as a series of steps orchestrated by the platelets (George 2000).

The first step of primary haemostasis follows vessel injury exposing collagen fibres that immobilise von Willebrand factor (vWF) molecules. Blood flow unfolds vWF from a globular circulating state and *platelet adhesion* occurs via GpIbα and platelet collagen receptors (integrin α2β1 and GpVI). Adhesion prompts transmembrane signal induction and a series of *platelet activation* events including degranulation to release ADP, calcium, coagulation factors, thromboxane A2 and serotonin (George 2000). The activated platelets undergo cytoskeletal changes, spreading to provide a negatively charged platform for coagulation complex assembly. Activated and adherent platelets expose the $\alpha_{IIb}\beta_3$ complex allowing vWF and fibrinogen-mediated *platelet aggregation* (George 2000).

Another hurdle is characterising this complex process, as available laboratory techniques have significant limitations (Haley et al. 2014). Aggregometry is a reasonably accessible and more frequently used technique, measuring changes in impedance or turbidity to reflect platelet activation by agonists (e.g. ADP, collagen, ristocetin, arachidonic acid, thrombin-reactive peptide). Aggregometry studies indicate that platelets derived from cord blood of term babies have reduced responsiveness to agonists compared to those of adults (Sola-Visner 2012; Israels et al. 1990). However, large volumes of blood are required for aggregometry, limiting its use to the study of cord blood-derived platelets, which may not be functionally equivalent to those derived from postnatal peripheral blood (Del Vecchio et al. 2015; Saxonhouse et al. 2010).

Flow cytometry has the advantage of using smaller sample volumes (Del Vecchio et al. 2015). Following application of platelet agonists, monoclonal antibodies can be used to quantify the expression of activation markers such as P-selectin. This technique supports evidence from aggregometry studies, with platelets from peripheral blood of both term and preterm babies showing comparative hyporesponsiveness (Rajasekhar et al. 1994, 1997; Del Vecchio et al. 2015; Bednarek et al. 2009). This is more pronounced at lower gestational age (Sitaru et al. 2005), and the effect persists into the second week after birth (Bednarek et al. 2009; Sitaru et al. 2005; Rajasekhar et al. 1997).

Evidence for a hyporesponsive platelet phenotype in neonates might encourage clinicians to be more cautious in thrombocytopenic neonates and raise platelet transfusion thresholds. However, aggregation and flow cytometry only assesses a particular element of platelet function, not the entire complex process of primary haemostasis. Both technologies also fail to reflect the physiological conditions under which it occurs. For example, in vivo, factors such as the haematocrit, blood proteins and shear stress are known to modulate primary haemostasis (Israels et al. 2001; Shenkman et al. 1999).

The platelet function analyser (PFA-100) attempts to mimic flow and shear stress by aspirating blood through a narrow aperture in a membrane coated with either collagen and ADP or epinephrine. Time to aperture closure provides an assessment of platelet aggregation. Studies using this technique demonstrate shorter closure times in cord blood samples from term neonates compared to adults suggesting enhanced primary haemostasis (Israels et al. 2001). This is replicated in peripheral blood samples, although the effect is less pronounced than in cord blood, highlighting a possible difference in the functionality of platelets derived from each source (Saxonhouse et al. 2010). Supporting studies using flow chambers measure platelet adhesion to a coated surface under arterial shear stresses and also demonstrate increased deposition of neonatal platelets on an extracellular matrix (Shenkman et al. 1999). This appears to be mediated by higher concentrations of vWF in neonatal plasma and a higher concentration of the highest-molecular-weight multimers compared to adult blood (Weinstein et al. 1989). These laboratory studies indicating enhanced primary haemostasis are supported by studies demonstrating shorter bleeding times in healthy term and preterm babies in comparison to adults (Andrew et al. 1990; Del Vecchio et al. 2008).

In summary, measures of primary haemostasis in neonates indicate that it is comparatively enhanced compared to adults. Therefore, despite the hyporesponsive phenotype of neonatal platelets seen with aggregation and flow cytometry studies, it can be concluded that the apparent 'immaturity' of neonatal haemostasis probably does not, in itself, justify raised platelet transfusion thresholds for neonates with thrombocytopenia.

Thrombocytopenia in Clinical Practice

In the vast majority of newborns with thrombocytopenia, low platelet counts are largely the consequence of other significant morbidities, for example, prematurity, infection and/or intrauterine growth restriction. It is only for the rare, specific diagnoses, such as fetal and neonatal alloimmune thrombocytopenia (FNAIT), thrombocytopenia due to maternal autoimmune thrombocytopenia (ITP) or the

hereditary thrombocytopenias, which the baby may present with thrombocytopenia and its sequelae alone.

Low platelet counts in the newborn baby may be the consequence of increased consumption, decreased production or rarely sequestration. But unlike in older children or adults, immaturity of thrombopoiesis is likely to be a common contributing factor, particularly in preterm infants. The multifactorial nature of thrombocytopenia in the newborn has meant that it has become conventionally classified by its time of onset (Table 1): fetal if already present at birth; early onset if detected soon after or developing within 72 h of birth; late onset if it develops after the immediate postnatal period. Within each category, there are specific causes, and many aetiologies follow a consistent time course. There are, in addition, causes that may present at any time in the neonatal period.

Fetal Thrombocytopenia

The most severe in utero thrombocytopenia is associated with FNAIT. Hohlfeld and colleagues (1994), in their study of over 5000 fetal blood samples, showed fetuses with FNAIT have a mean platelet count of 51×10^9/L (range 3–145 $\times$ 10^9/L). There is a high risk of intracranial haemorrhage and intrauterine death.

Haemolytic disease of the newborn, particularly when due to rhesus isoimmunisation, is also often associated with thrombocytopenia. It is likely to be secondary to fetal hypoxia driving compensatory erythropoiesis at the expense of other lineages, including megakaryocytes, as demonstrated by Koenig and Christensen (1989a), who found reduced megakaryocyte production in affected fetuses. Other fetal aetiologies are more commonly not identified until after birth and present as early-onset thrombocytopenia.

Early-Onset Thrombocytopenia

Early thrombocytopenia, defined as developing within 72 h of birth, accounts for the majority (up to 75 %) of thrombocytopenic episodes in the newborn. In most, the platelet count continues to fall after birth, reaching a nadir by day 4 or day 5 (Fig. 1); thereafter the platelet counts of most babies start to recover by day 7, and more than 85 % have normal platelet counts by the end of the second week (Castle et al. 1986; Metha et al. 1980; Christensen et al. 2006). The thrombocytopenia is usually mild to moderate, the platelet count rarely falling below 50×10^9/L, and in the majority does not require platelet transfusion support. In more severely affected babies it may persist, especially if a further insult to the fragile thrombopoiesis supervenes.

The most clearly defined cause of early-onset thrombocytopenia is placental insufficiency and chronic fetal hypoxia, commonly associated with maternal pre-eclampsia, hypertension, diabetes and fetal growth restriction. Several groups have shown that the mechanism is impaired megakaryocytopoiesis, with reduced circulating megakaryocyte progenitors and megakaryocytes and associated with raised Tpo at birth (Murray and Roberts 1996; Watts et al. 1999; Watts and Roberts 1999) (Fig. 2). These babies frequently have other associated haematological abnormalities, such as neutropenia, polycythaemia and nucleated red cells in the peripheral circulation at birth (Philip and Tito 1989; McIntosh et al. 1988). The neutropenia has also been shown to be secondary to impaired granulocytopoiesis (Koenig and Christensen 1989b, 1991). Babies born under these conditions have been shown to have raised erythropoietin levels (Salvesen et al. 1993), leading researchers to conclude that the constellation of abnormalities are due to hypoxia-related EPO-driven excess of erythropoiesis from multipotent

Table 1 Causes of *acquired* neonatal thrombocytopenia

Onset	Impaired production	Consumption
Fetal	Congenital infection (e.g. CMV, toxoplasma, rubella, parvovirus) Bone marrow replacement, e.g. congenital leukaemia, neuroblastoma Rhesus disease	Congenital infection (e.g. CMV, toxoplasma, rubella) Alloimmune (FNAIT) Autoimmune (maternal ITP, SLE) Haemophagocytic lympho-histiocytosis (HLH)
Early onset	Chronic fetal hypoxia (e.g. pregnancy-induced hypertension, intrauterine growth restriction, maternal diabetes) Congenital infection (e.g. CMV, toxoplasma, rubella, parvovirus) Metabolic disease	Perinatal hypoxia-ischaemia (DIC-related) Perinatal bacterial or fungal infection Congenital infection (e.g. CMV, toxoplasma, rubella, parvovirus) Alloimmune (FNAIT) Autoimmune (e.g. maternal ITP, SLE) Kasabach-Merritt syndrome (secondary to haemangiomas)
Late onset	Prolonged thrombocytopenia after sepsis and necrotising enterocolitis Congenital infection Metabolic disease	Late-onset sepsis Necrotising enterocolitis Acquired viral infection (e.g. CMV) Congenital infection Kasabach-Merritt syndrome

CMV cytomegalovirus, *FNAIT* fetal and neonatal alloimmune thrombocytopenia, *ITP* immune thrombocytopenia, *SLE* systemic lupus erythematosus

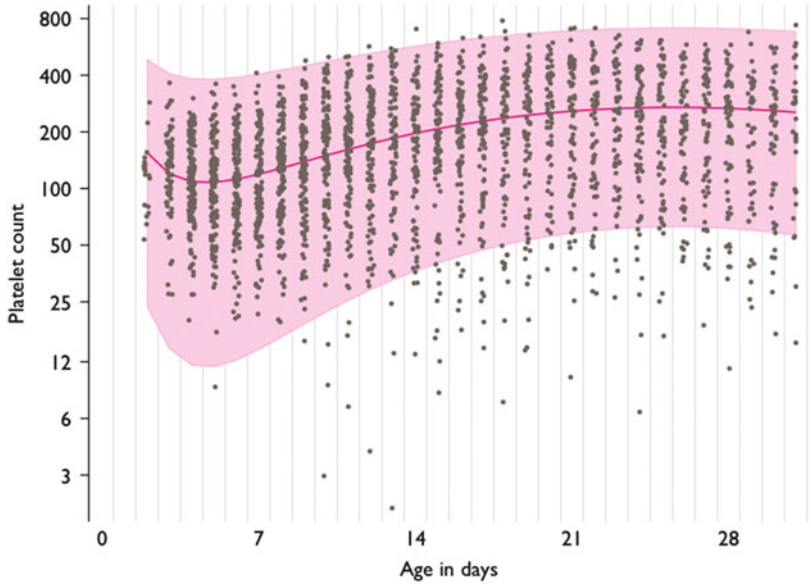

Fig. 1 Sequential daily platelet counts from a cohort of 141 small for gestation preterm infants, demonstrating the dynamic changes in peripheral blood platelet numbers during the first postnatal month. The median and 95 % reference range is shown. (*Published: Carr, Kelly, Williamson. Neonatology 2015*)

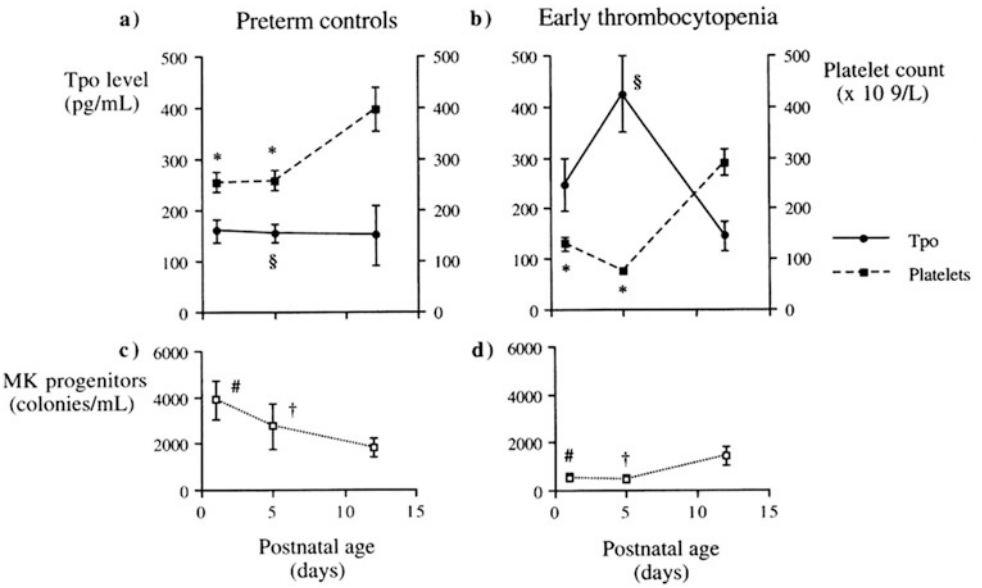

Fig. 2 Serial plasma thrombopoietin (Tpo), platelets and megakaryocyte (MK) progenitors in 28 preterm babies from uncomplicated pregnancies with and without early thrombocytopenia. (*Published: Watts TL, Murray NA, Roberts IAG. Pediatric Research 1999*)

progenitor cells, at the expense of the neutrophil and megakaryocyte lineages (the so-called lineage-steal hypothesis). That this may occur in fetal and early neonatal life, but not in other clinical circumstances of EPO excess, provides additional evidence that the capacity of the progenitor pool may be more limited in the fetus and preterm infant, as outlined in the above section on fetal megakaryocytopoiesis.

Christensen et al. (2006) found that the cause of thrombocytopenia could not be identified in almost 50 % of the babies in their study of extremely low birth weight (birth weight <1000 g) newborns. This is also the experience of most neonatologists. Early-onset thrombocytopenia is most frequent in the most prematurely born preterm babies in their first few days, when they are at their most unstable. It has been described in association with more general factors that are likely to be indicators of severity of illness in premature babies, such as antepartum haemorrhage, umbilical arterial or venous catheter insertion, respiratory distress syndrome, mechanical ventilation and phototherapy for hyperbilirubinaemia.

It seems likely that otherwise unexplained, early-onset thrombocytopenia is a final common outcome from multiple insults in the sick newborn: (1) underlying vulnerable megakaryopoiesis, with developmental difficulty in upregulating platelet production; (2) impairment in megakaryocytopoiesis in babies with placental insufficiency; (3) systemic inflammation related to perinatal infection or chorioamnionitis in the absence of frank infection; (4) frequent phlebotomy in babies with high numbers

of circulating megakaryocyte progenitors might also deplete reserves for platelet production.

Late-Onset Thrombocytopenia

Late-onset thrombocytopenia is most commonly associated with acquired systemic infection or necrotising enterocolitis. The presentation follows a distinct pattern, frequently of sudden onset and is often severe, requiring at least one and often many platelet transfusions (Zipursky et al. 1976; Mondanlou and Ortiz 1981; Baer et al. 2009). It usually has a self-limiting course, commonly lasting 5–7 days, although it may be prolonged and persist even after the precipitating cause is treated (Murray 1999; Roberts and Murray 2003). Babies who have had significant early thrombocytopenia may be more likely to have persistent thrombocytopenia if they develop additional late thrombocytopenia with intercurrent sepsis. This may be related to reduced megakaryocytopoiesis (Cremer et al. 2013).

Late-onset bacterial infection occurs in 25–41 % of very low birth weight (<1500 g) babies (Stoll et al. 1996; Carr et al. 2009), and thrombocytopenia accompanies bacterial infection in 50–75 % of these episodes, irrespective of causative organism (Baer et al. 2009; Carr et al. 2015). Postnatally acquired viral infections may also precipitate thrombocytopenia, though CMV is now a less frequent cause where the policy is to give only CMV-negative or leucodepleted blood products. Necrotising enterocolitis (NEC), a condition occurring in low birth weight preterm infants and characterised by ischaemic bowel necrosis but with uncertain aetiology (Lin and Stoll 2006), precipitates thrombocytopenia in more than half of cases which can be particularly severe and refractory (Hutter et al. 1976; Kenton et al. 2005; Baer et al. 2009; Stanworth et al. 2009).

Thrombocytopenia Associated with Thrombosis or Coagulopathy

Consumptive thrombocytopenia may occur at any time, associated with activation of the clotting cascade leading to local thrombosis or disseminated intravascular coagulation.

Thrombotic events are relatively common in the newborn period and are frequently associated with a consumptive thrombocytopenia. The most common presentation is secondary to large vessel thrombosis from vascular access devices such as umbilical arterial or venous lines, usually in the preterm baby. Spontaneous thrombosis may also occur, more commonly in babies with predisposing factors such as perinatal hypoxia-ischaemia and polycythaemia. Renal vein thrombosis is an example of this and classically presents with the triad of a renal mass, haematuria and thrombocytopenia. Treatment is often expectant, due to concerns of haemorrhage in newborn period, although thrombolysis and anticoagulation are used in some circumstances.

Neonatal thrombocytopenia may also be associated with concomitant coagulopathy typical of disseminated intravascular coagulation (DIC), the most common circumstance being in severe perinatal hypoxia-ischaemia. But evidence is often lacking for the presence of DIC in many episodes of thrombocytopenia, and it appears to be a contributory factor in only a minority (10–20 %) (Castle et al. 1986; Metha et al. 1980; Gross et al. 1982). Although DIC is often cited as being associated with neonatal sepsis, there are a number of studies that suggest this is commonly not the case (Zipursky et al. 1976; Corrigan 1974; Aronis et al. 1998). In necrotising enterocolitis, DIC has been shown to be present in only about one in four babies affected (Sonntag et al. 1998).

Immune Thrombocytopenias

Fetal and Neonatal Alloimmune Thrombocytopenia (FNAIT)

FNAIT occurs when the mother forms an antibody to a fetal human platelet antigen (HPA) inherited from the father. The antibody crosses the placenta, initiating fetal platelet destruction. This condition is exactly analogous to haemolytic disease of the newborn (HDN). Unlike the majority of disease states associated with neonatal thrombocytopenia, FNAIT carries a significant risk of severe haemorrhage as a consequence of the antibody also interfering with platelet function (Ghevaert et al. 2007; Williamson et al. 1998).

Half of HPA1a-positive babies born to HPA1a-immunised mothers are thrombocytopenic ($<150 \times 10^9$/L), and 50 % of those have platelet counts of less than 20×10^9/L at birth. A low platelet count increases the risk of major and life-threatening haemorrhage, but this is variable depending on the antibody specificity (Ghevaert et al. 2007; Williamson et al. 1998).

Incompatibility between maternal and fetal HPA occurs in 1 in 350 pregnancies, but FNAIT only occurs in about 1 in 1000 live births (Curtis 2015; Turner et al. 2005). There have been 36 HPA antigens described so far that have been shown to lead to antibody production causing FNAIT (Curtis 2015). HPA1a is the commonest antigen, causing 80–85 % of cases in white populations, but rarely in non-white. Platelets are negative for HPA1a in 2–2.5 % of Caucasians (Williamson et al. 1998; Davoren et al. 2004; Knight et al. 2011). The polymorphic variant is carried on the β3 subunit of the $\alpha_{IIb}\beta_3$ integrin, which is the major fibrinogen receptor and is involved in platelet activation and aggregation responses

that are central to platelet thrombus formation. It is the involvement of HPA antigens in such crucial platelet functions that are likely to be the reason that these babies so frequently have bleeding complications associated with their thrombocytopenia. Other common antigens include HPA5b, seen in 10–15 % of cases of FNAIT, and which is more common in African populations. Antibodies with anti-HPA-5b specificity appear to increase the risk of haemorrhage. In one series, four cases with this specificity had intracranial haemorrhage at a platelet count that was either in the normal range or considered to be safe (Ghevaert et al. 2007).

Unlike haemolytic disease of the newborn, FNAIT commonly develops during first pregnancies (up to 50 % of the time) often in the absence of obvious isoimmunisation events such as fetomaternal bleeding. This raises the question as to how sensitisation occurs. There is evidence that it may be from shedding of trophoblasts from fetal chorionic villae in the placenta into the maternal circulation, as these cells express integrin beta3, that also forms part of the glycoprotein that contains HPA1a on platelets (Curtis 2015; Kumpel et al. 2008). Another key feature of this condition is the strong association between the formation of the antiplatelet antibodies and maternal expression of the HLA class II antigen DRB3*01:01, present in 99 % of anti-HPA1a-producing women (Williamson et al. 1998; Bussel 2009). It appears that DRB3*01:01 enhances HPA1a antigen presentation and the immune response to it (Curtis 2015).

A fetus affected by FNAIT may develop thrombocytopenia from as early as 20 weeks gestation. In untreated pregnancies, 10–20 % develop intracranial haemorrhage which may lead to intrauterine death or significant disability and a third resulting in cerebral palsy (Roberts et al. 2008; Sharif and Kuban 2001; Ward et al. 2006). In an important minority of cases, intracranial haemorrhage may occur postnatally; hence early-diagnosis essential and postnatal platelet support is of critical importance.

The mainstay of management of a baby with FNAIT in the newborn period is to transfuse with HPA-compatible platelets. Both the baby and mother should be HPA typed and the mother tested for alloantibodies. The National Blood Service in the UK keeps stocks of platelets negative for HPA1a or HPA5b on the shelf ready for urgent use. If appropriately matched platelets are not immediately available, pooled un-typed platelets should be given. As the risk of bleeding is high in this condition, standard practice is to keep the platelet count above 50, or 100×10^9/L in the presence of significant bleeding. If repeated transfusions do not achieve reasonable platelet increments, intravenous immunoglobulin (IVIG) should be added (te Pas et al. 2007). A cranial ultrasound must always be performed at presentation.

In subsequent pregnancies, excellent outcomes have been achieved with active management. Because of the dangers of intrauterine cordocentesis and transfusion, the accepted treatment is now the provision of weekly IVIG from 12 to 20 weeks of gestation, with careful ultrasound monitoring for evidence of fetal bleeding. Later in gestation, steroid therapy may be added (van den Akker et al. 2007; Berkowitz et al. 2006; Pacheco et al. 2011).

There has been interest in recent years in the development of a prophylactic treatment (Curtis 2015). This is analogous to anti-D antibody prophylaxis in rhesus haemolytic disease, where the antibody administered to the mother induces antibody-mediated immune suppression, leading to failure of the mother to produce destructive red cell antibodies, most likely through rapid clearance of D-positive cells from the maternal circulation.

An FNAIT prophylaxis programme requires both screening mothers for an at-risk HPA type and an effective anti-HPA antibody. Most research into this has looked at HPA1a, screening for pregnant women who are HPA1a negative. Screening can now be done rapidly and reasonably cost-effectively (Garner et al. 2000). As far as prophylactic anti-HPA1a antibody is concerned, there are a number of approaches being actively investigated (reviewed: Curtis 2015). A number of groups have worked with animal models which show promise; a recent study in healthy volunteers of a recombinant anti-HPA1a IgG antibody showed reduced clearance of platelets following antibody administration, suggesting this may be a suitable model for prophylaxis, and an international collaborative project is looking at purifying large quantities of anti-HPA1a antibody from positive women previously immunised by NAIT-affected pregnancies. Should sufficient antibody be obtained, prophylaxis trials are planned (Kjeldsen-Kragh et al. 2007; Kjelsen-Kragh et al. 2012).

Neonatal Autoimmune Thrombocytopenia

Autoantibodies against maternal platelets that cause maternal thrombocytopenia are present in 1-2 per 1000 pregnancies (Gill and Kelton 2000; Kelton 2002). The most common clinical circumstances are in maternal ITP and SLE. In maternal ITP only 10 % of babies develop thrombocytopenia. Major bleeding complications are rare, with intracerebral haemorrhage thought to develop in no more than 1 % (Burrows and Kelton 1990b; Valat et al. 1998). As neonatal bleeding is not likely, there is no necessity to counsel against vaginal delivery for the baby's well-being. The severity of fetal and neonatal thrombocytopenia in ITP tends to correlate with the severity of maternal thrombocytopenia and is consistent in subsequent pregnancies (Valat et al. 1998).

When maternal thrombocytopenia is known to be associated with autoimmune disease, all babies should

have platelet counts measured on cord blood samples or from the baby soon after birth. If the platelet count is normal, then no further testing is necessary. If the platelet count is $<150 \times 10^9$/L at birth, repeat testing should be carried out over subsequent days as the platelet count will often fall further. The usual natural history of the thrombocytopenia is for the platelets to then recover by 7–10 days after birth. The treatment threshold should be according to the standard guidelines (Table 3), with IVIG recommended as first line, unless there is significant bleeding in which case pooled platelets can be given (Ballin et al. 1988).

Thrombocytopenia and Clinical Bleeding

Newborns, particularly preterm infants, are at high risk of bleeding, but the evidence implicating thrombocytopenia directly in most bleeding episodes in the newborn period is conflicting. Haemostasis depends on the complex interplay between platelets (number and function), vascular physiology and coagulation. All aspects of this triad are potentially vulnerable in the newborn period, and the relative contribution of a low platelet count is far from clear.

Preterm neonates frequently develop significant bleeding complications in the absence of thrombocytopenia and coagulopathy. It is likely that some episodes are more the consequence of vascular fragility and haemodynamic instability. For example, the pathogenesis of both intraventricular and pulmonary haemorrhage is often associated with reperfusion injury after a hypoxic-ischaemic insult related to perinatal or postnatal illness.

Attempts to define the uncertain relationship between a low platelet count and clinically significant bleeding in the newborn have been hampered by lack of an agreed classification of bleeding severity, and no consistent definition of severity of thrombocytopenia. These uncertainties were addressed in a systematic way for the first time by the UK PlaNeT-1 study.

PlaNeT-1 was a prospective, year-long observational cohort study involving seven regional neonatal intensive care units in the UK. The study documented platelet counts and bleeding episodes occurring in all babies whose platelet counts fell below 60×10^9/L. Episodes of bleeding were recorded, based on a consistent classification scale, along with contemporaneous platelet counts and platelet transfusions (Stanworth et al. 2009; Muthukumar et al. 2012; Venkatesh et al. 2013).

Five percent of all babies admitted to the study of neonatal units developed a platelet count $<60 \times 10^9$/L. One third (34 %) of babies whose platelet count fell below 60×10^9/L subsequently had a nadir count of less than 20×10^9/L. Only 9 % developed bleeding classified as severe. Minor haemorrhage was common (73 %), but 18 % of babies had no haemorrhage of any severity recorded. Although these data suggest that minor haemorrhage is a frequent consequence of severe thrombocytopenia, nadir platelet counts were actually highest in the babies with major haemorrhage and lowest in babies with no haemorrhage recorded.

An important and novel finding of the PlaNeT-1 study was that major haemorrhage was most strongly associated with lower gestational age, and a postnatal age less than 2 weeks. The most common episodes of minor haemorrhage described were haematuria, bleeding from the endotracheal tube, nasogastric tube and skin. The insights gained from the PlaNeT-1 study have also led to the development of a more consistent classification of bleeding severity for use in future treatment trials (Table 2) (Curley et al. 2014).

In summary, (1) the degree of thrombocytopenia was not a useful predictor of haemorrhage risk, and (2) there was no evidence that minor bleeding episodes predicted the development of major haemorrhage.

Table 2 Definitions of bleeding grades

Minor	Any bleed from the skin, umbilical cord, skin around stoma, surgical scar, mucosa Pink frothy secretions or old blood from the endotracheal tube Cranial USS: germinal matrix haemorrhage (Papile grade 1)
Moderate	Frank blood from stoma Macroscopic haematuria Cranial USS: intraventricular haemorrhage without ventricular dilatation (Papile grade 2) Fresh blood from endotracheal tube, without ventilatory changes
Major	Macroscopic rectal blood Fresh blood from endotracheal tube with ventilator changes Cranial USS: intraventricular haemorrhage with ventricular dilatation (Papile grade 3) or with parenchymal infarction (Papile grade 4)
Severe	Life-threatening major bleed with haemodynamic instability requiring volume boluses or red cell transfusion within 24 h Fatal major bleeding

USS ultrasound scan, *ETT* endotracheal tube, *IVH* intraventricular haemorrhage
Reference: Curley et al. (2014)

It should be noted that none of the babies described in the PLaNeT study had a diagnosis of FNAIT. It is clear that FNAIT, in which impaired platelet function coexists with severe thrombocytopenia, is frequently complicated by life-threatening haemorrhage (Ghevaert et al. 2007; Williamson et al. 1998).

Platelet Transfusion

Indications

Platelet transfusion is common in neonatal practice, but the evidence base to guide the use and thresholds of platelet transfusion is poor (Roberts et al. 2008; Christensen 2011; Curley et al. 2014). Neonatologists are understandably fearful of the consequences of bleeding and will give prophylactic transfusions in non-bleeding babies to avoid this (Josephson et al. 2009; Del Vecchio et al. 2001). Surveys in both the UK and the USA have shown that there is a wide range of transfusion thresholds used between units and between practitioners, with thresholds generally higher in the USA than Europe (Chaudhary and Clarke 2008; Josephson et al. 2009; Cremer et al. 2011).

Surprisingly, no studies have provided evidence of clinical benefit from platelet transfusion, or a clear link with fewer bleeding complications. Indeed there are several studies that have correlated a high number of platelet transfusions to excess morbidity and mortality in preterm babies (Del Vecchio et al. 2001; Kenton et al. 2005; Baer et al. 2007). Although the explanation for this may well be that sicker babies receive more platelet transfusions, it would be wise to sound a note of caution in the excessively liberal use of platelet transfusions in non-bleeding babies.

There are a number of consensus guidelines for platelet transfusion that have been published. The British Committee for Standards in Haematology published guidance in 2004, which relate transfusion decisions to platelet thresholds and clinical stability (Table 3) (Gibson et al. 2004). These guidelines are currently undergoing revision.

The only randomised controlled trial of prophylactic platelet transfusion at different thresholds was published over 20 years ago (Andrew et al. 1993). This study randomised babies to have their platelet count kept above 50 or above 150 $\times$ 10^9/L and showed no benefit in keeping platelets at the higher threshold with regard to any outcomes including bleeding. However, this study no longer reflects current practice, as many neonatologists use a prophylactic platelet transfusion threshold well below 50 $\times$ 10^9/L. The UK guideline (Gibson et al. 2004) suggests a threshold for preterm babies without clinical haemorrhage of 30 $\times$ 10^9/L and term babies of 20 $\times$ 10^9/L (Table 3).

A randomised controlled study is, at the time of writing, underway in the UK with 50 participating neonatal units. The Platelets for Neonatal Transfusion Study 2 (PlaNeT-2) is designed to compare the safety of a higher or lower transfusion threshold (Curley et al. 2014). This study randomises non-bleeding preterm neonates <34 weeks of gestation at birth to receive platelet transfusions below the threshold of either 25 $\times$ 10^9/L or 50 $\times$ 10^9/L. It is a reflection of existing variability in platelet transfusion practice and lack of supportive evidence that during the planning stage, concerns were expressed in equal measure, that the lower threshold placed babies at too high a risk of bleeding, and that the upper threshold was too conservative and would expose babies to excessive transfusion.

An alternative approach to the use of a simple platelet count as the arbiter of whether to give a platelet transfusion has been investigated in a study that takes into account the effect that platelet size has on platelet function and the effectiveness of the platelet plug (Gerday et al. 2009). In this study babies were randomised to the use of the 'platelet mass index', being the product of the platelet count and the mean platelet volume, compared to the platelet count alone for deciding whether to transfuse platelets. It was found that babies in the platelet mass directed transfusion group had fewer transfusions, with no detectable difference in bleeding outcomes.

A more extensive review of transfusion practice, and the practical provision of platelets for transfusion to newborn infants, is covered in a recent review (Carr et al. 2015).

Table 3 UK Platelet transfusion guidelines

Platelet count	First postnatal week Without significant haemorrhage	After first week Without significant haemorrhage	Any postnatal age With significant haemorrhage[a]
<20–30 $\times$ 10^9/L	Transfuse all infants	Transfuse all infants	Transfuse all infants
<50 $\times$ 10^9/L	Transfuse if high risk[b]	Do not transfuse	Transfuse
50–100 $\times$ 10^9/L	Do not transfuse	Do not transfuse	Transfuse
>100 $\times$ 10^9/L	Do not transfuse	Do not transfuse	Do not transfuse

[a]Significant haemorrhage includes grade 2+ intraventricular haemorrhage, pulmonary haemorrhage with respiratory deterioration and gastrointestinal bleeding (Some clinicians may treat 'moderate' haemorrhage as 'significant')

[b]High risk includes birth weight <1000 g, gestation <28 weeks, clinical instability, previous haemorrhage, abnormal coagulation, systemic sepsis or necrotising enterocolitis

Reference: Gibson et al. (2004)

Risks and Complications

Platelet transfusion, in common with the use of other blood products, is not without risk. Adverse incidents that involve the transfusion of blood products are relatively common. Data from the UK Serious Hazards of Transfusion (SHOT) national haemovigilance scheme shows that the there is a disproportionate burden of incidents in the newborn population, with 16 % of reported incidents occurring to neonates <28 days of life (Stainsby et al. 2008). Most of these reports are from practical errors. Infection is also well described. Bacterial contamination is more likely with platelets than with other products, and transfusion-transmitted viral and other infections are also clearly a risk (Baer et al. 2007; Curley et al. 2014).

Alternatives to Platelet Transfusion

Thrombopoietin Analogues

Tpo analogues are used in a variety of clinical settings in thrombocytopenic adults, and they have been approved for use in the USA for treatment of severe refractory immune thrombocytopenia (Kuter 2010; Journeycake 2012; Arnold 2013). There has been interest in the idea of using Tpo analogues to reduce the incidence or severity of neonatal thrombocytopenia (Sallmond et al. 2010; Ferrer-Marin et al. 2010).

There are several reasons why this approach is attractive. Many episodes of neonatal thrombocytopenia are severe and prolonged, and these babies may have very many transfusions. There is evidence for a developmental limitation in the newborn to the megakaryopoietic response to stress. Some researchers have concluded that there may in addition be a suboptimal Tpo response to thrombocytopenia, which may be particularly relevant in babies with early thrombocytopenia and reduced megakaryocytopoiesis. Conversely, neonatal progenitors appear to be more sensitive to Tpo stimulation, so may be an effective target for therapeutic stimulation.

There are, however, many factors that raise doubts about this form of therapy entering standard neonatal practice. Most important is the timeframe for an adequate increase in peripheral blood platelets following Tpo analogue treatment, which is unlikely to be rapid enough to be useful for the majority of thrombocytopenic episodes. In adults, platelets start to increase at 4–6 days but only reach a peak at 10–14 days after treatment (Kuter 2010). By this time most neonatal thrombocytopenic episodes will have already resolved spontaneously. Differences between newborn and adult megakaryocytopoiesis may prolong this response time further in the newborn.

There are other reasons for caution. Initial studies of recombinant Tpo led to the development of neutralising antibodies which cross-reacted with endogenous Tpo, leading to refractory thrombocytopenia and aplastic anaemia, although more recent synthetic analogues appear not to induce antibodies. The analogous use of the neutrophil cytokines, GM-CSF and G-CSF, for stimulation granulopoiesis to prevent or treat neutropenia and so reduce sepsis has not translated into clinical benefit (Carr et al. 2009; Kuhn et al. 2009; Carr 2013). Tpo receptors are present on some non-haemopoietic cells, including in the nervous system, raising the possibility of unintended toxicities following their use in the newborn (Dame et al. 2003), and there have been studies that have shown that Tpo may have proapoptotic and differentiation-blocking effects on the developing brain (Ehrenreich et al. 2005; Samoylenko et al. 2008). Finally, the effects of potent biological simulators of haemopoietic stem cells early in life raise anxieties about unpredictable long-term consequences, though this has not occurred following the now extensive use of erythropoietin or of G- and GM-CSF in newborns.

None the less, prolonged thrombocytopenia is a clinical challenge in a number of preterm infants, and for these Tpo therapy might be effective and lead to clinical benefit. More research and clinical trials in the area are warranted.

Donor-Independent Platelets for Transfusion

A number of research groups are investigating the possibility of producing megakaryocytes and platelets ex vivo, with the goal of donor-independent platelet transfusions, with improvements in safety and quality. The aim is to produce self-sustaining cultures of stem cells, primitive or more mature megakaryocyte progenitor cells, that will continuously produce megakaryocytes and platelets for transfusion, obviating the need for platelet donation. Although various cell lines have been successfully established, current obstacles to the development of these systems include difficulties with sufficient yield of platelets for clinical application (Lambert et al. 2013). However, the small blood volumes of preterm infants and their added vulnerability to multiple donor exposure would make them ideal early beneficiaries.

Conclusion

Thrombocytopenia in the neonatal period is common. It most frequently occurs in extremely preterm babies but is often only mild to moderate and self-limiting. However, concerns about the high risk of bleeding and the potential major sequelae of death or neurodisability are strong drivers for replacement therapy, with frequent and repeated platelet transfusions.

The underlying cause of neonatal thrombocytopenia in the preterm population is usually multifactorial. As increasingly premature infants survive, the various patterns of thrombocytopenia related to common clinical circumstances have been defined by careful clinical observation. Now there is need for greater understanding and definition of normal developmental physiology to underpin prevention and treatment strategies to reduce the risk and consequences of too few platelets (Box 1).

Box 1: Future Research Perspectives

Aetiology

Biology and homeostasis of fetal/neonatal megakaryopoiesis

Mechanisms of thrombocytopenia, in particular the pathogenesis of:

Early thrombocytopenia in the absence of placental dysfunction

Prolonged postnatal thrombocytopenia

Clinical

Standardisation of platelet function assays and establishing neonatal normal ranges

Defining clinically appropriate platelet transfusion thresholds

Therapies

Thrombopoietin for prevention or treatment of thrombocytopenia

Antibody prophylaxis for fetal and neonatal alloimmune thrombocytopenia (FNAIT)

Donor-independent platelets for transfusion

In the meantime, the mainstay of treatment in neonatal thrombocytopenia remains platelet transfusion, and careful ongoing studies to define safe transfusion thresholds are important to achieve consensus on clinical management. However, we can also look forward to novel treatments: antibody prophylaxis for mothers at risk of developing the alloantibodies responsible for neonatal alloimmune thrombocytopenia, thrombopoietin or other agents to enhance endogenous platelet production and the prospect of off-the-shelf in vitro platelets produced in quantities sufficient for small babies.

Take-Home Messages

- A normal platelet count at birth is $>150 \times 10^9$/L.
- Thrombocytopenia is most common in preterm infants, in whom the progenitor pool appears limited.
- Neonatal platelets have an immature functional phenotype as measured by aggregometry, but in vitro and clinical measures of primary haemostasis which indicate enhanced haemostatic activity compared with adults. Available evidence on function does not justify raised platelet transfusion thresholds.
- The relationship between platelet count and clinical bleeding risk remains poorly defined, with no international consensus on appropriate transfusion thresholds.
- Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a clearly defined syndrome associated with life-threatening haemorrhage caused, uniquely, by antibodies with specificity for functional platelet antigens.
- There is ongoing research into alternatives to donor platelets, such as thrombopoietin analogues, but platelet transfusion to prevent bleeding is likely to remain the mainstay of management for the foreseeable future.

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Thrombocytopenia in Patients with Solid Tumors or Hematologic Malignancies

Caroline I. Piatek and Howard A. Liebman

Abstract

Thrombocytopenia is a common finding in patients with cancer and is most frequently the result of systemic cancer treatment. The mechanism by which cancer therapies cause thrombocytopenia is varied, leading to differing incidences and patterns of thrombocytopenia. In addition to megakaryocyte cytotoxicity, various cancer drugs have been associated with immune thrombocytopenia, thrombotic microangiopathy, and splenomegaly. Decreased platelet production due to direct tumor involvement of the bone marrow is more common in patients with hematologic malignancies compared to those with solid tumors. Splenic sequestration of platelets is an important cause of thrombocytopenia in both hematologic and solid cancers and may result from several different mechanisms. Disseminated intravascular coagulation is a common cause of thrombocytopenia in cancer patients and is associated with poor survival. Immune thrombocytopenia (ITP) is mostly often described in patients with chronic lymphocytic leukemia and also appears to be associated with inferior survival. Management of thrombocytopenia includes supportive care. Platelet transfusions are administered to patients with bleeding or at high risk of bleeding. The use of thrombopoietin receptor agonists may decrease the need for delays or dose reductions in patients receiving systemic cancer treatment. However, their use in this setting is still considered investigational.

Introduction

Thrombocytopenia is a frequent complication of cancer and may be due to a variety of causes. It is associated with an increased bleeding risk and increased healthcare costs (Wu et al. 2009; Liou et al. 2007). Thrombocytopenia may limit the delivery of cancer treatment on time and at the optimal dose, which may negatively impact patient outcomes (Elting et al. 2001; Bonadonna et al. 1995). Systemic cancer treatment is the most frequent cause of thrombocytopenia in cancer patients. Although megakaryocyte cytotoxicity is the most commonly recognized mechanism of thrombocytopenia resulting from cancer agents, the mechanism of thrombocytopenia varies. Other important causes of thrombocytopenia in cancer patients include direct tumor involvement of the bone marrow, splenomegaly, disseminated intravascular coagulation (DIC), and immune thrombocytopenia (ITP) (Table 1). Management includes supportive care with the administration of platelet transfusions for those with bleeding or at high risk of bleeding. Dose delays and dose reductions may be implemented with subsequent treatment cycles. Several small trials have explored the use of thrombopoietin (TPO) receptor agonists to mitigate thrombocytopenia in cancer patients receiving chemotherapy. However, their use is currently not considered to be standard.

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Table 1 Etiologies of thrombocytopenia in patients with cancer

Bone marrow involvement with malignancy
Splenomegaly
Systemic cancer treatment
Radiation therapy
Microangiopathic disorders
Immune thrombocytopenia

Thrombocytopenia Due to Direct Cancer Effect

Solid Tumor Involving the Bone Marrow

Bone marrow metastasis is uncommon in patients with solid tumors (Kopp et al. 2011). It signifies advanced disease and a dismal prognosis with a median survival of less than 2 months (Hung et al. 2014). Although reported with nearly all tumor types, the most common cancers to metastasize to the bone marrow are the stomach, lung, breast, and prostate (Chou et al. 2015).

Bone marrow infiltration by metastatic carcinoma can result in extramedullary hematopoiesis, predominantly involving the spleen. Premature splenic release of hematopoietic cells may lead to leukoerythroblastosis (nucleated red blood cells, tear drop red blood cells, and immature myeloid cells) in the peripheral blood. Therefore, in patients with unexplained cytopenias and/or leukoerythroblastosis, a bone marrow biopsy should be performed to evaluate for marrow infiltration by metastatic carcinoma (Fig. 1a, b).

Clinical manifestations of bone marrow metastases are nonspecific. Bone pain is the most common presenting symptom, occurring in 43–63 % of patients (Kim et al. 2007; Hung et al. 2014). Other common symptoms include fever (36 %) and bleeding (21 %) (Hung et al. 2014). Laboratory findings in the majority of patients include peripheral cytopenia, leukoerythroblastosis, and markedly elevated alkaline phosphatase (Hung et al. 2014). Thrombocytopenia is observed in 77 % of patients with bone marrow metastases, and platelet count less than 50×10^9/l is an independent predictor of inferior survival (Hung et al. 2014; Chou et al. 2015).

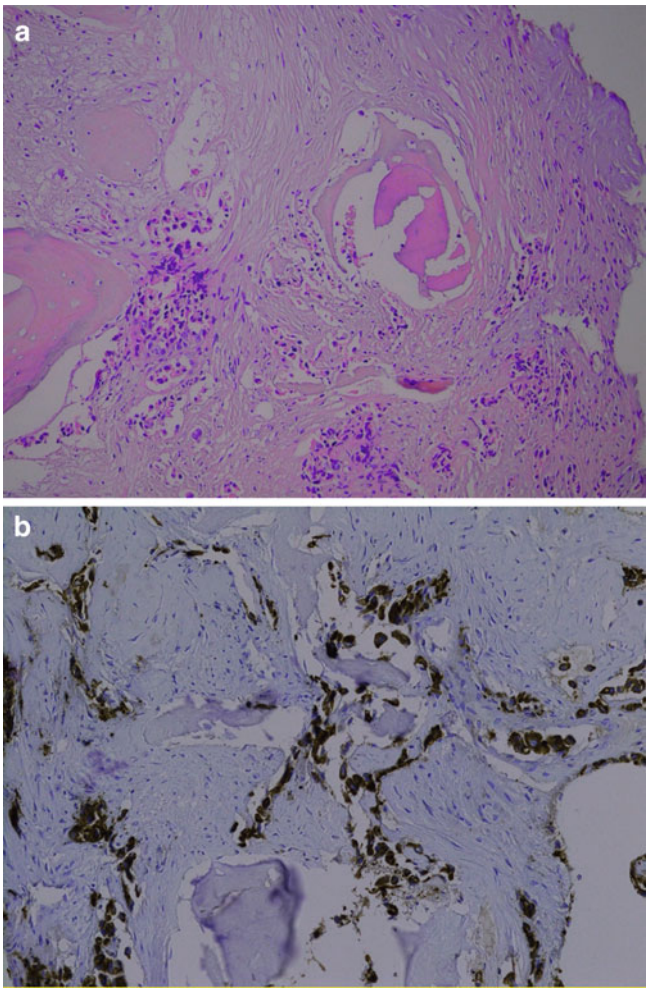

Fig. 1 (**a** and **b**) Bone marrow involvement with metastatic carcinoma of the breast. (**a**) Hematoxylin and eosin stain of a bone marrow trephine biopsy demonstrating clusters of pleomorphic cells with hyperchromatic nuclei embedded in a markedly fibrotic background. (**b**) Bone marrow trephine biopsy showing clustered AE1/AE3 positive carcinoma cells (*dark brown* staining) in a markedly fibrotic background

Hematologic Malignancies Involving the Bone Marrow

Thrombocytopenia is one of the most frequent laboratory abnormalities in hematologic malignancies. It is most often due to decreased platelet production as a result of bone marrow infiltration by malignant cells (Kuznetsov et al. 1992). Thrombocytopenia is incorporated into the staging system of a number of hematologic malignancies due to its negative prognostic significance. For example, a platelet count less than 100×10^9/L at the time of diagnosis of chronic lymphocytic leukemia (CLL) denotes advanced disease and is associated with a very poor outcome (Rai et al. 1975; Binet et al. 1981).

Splenomegaly Due to Tumor Involvement or Portal or Splenic Vein Thrombosis

One third of the total platelet mass is located in a spleen of normal size. With splenomegaly, 50–90 % of the total platelet mass is sequestered in the spleen, leading to a decrease in peripheral platelet counts (Aster 1966). Portal

hypertension is the most commonly recognized etiology of splenomegaly in patients with solid tumors. Portal hypertension may be due to a number of etiologies, including prehepatic portal hypertension (thrombosis, tumor invasion, or extrinsic compression by tumor), intrahepatic portal hypertension (cirrhosis or oxaliplatin-associated hepatic sinusoidal injury), or post-hepatic hypertension (Budd-Chiari syndrome). Splenomegaly also appears to be a common finding after resection of hepatic metastases. This phenomenon may result from the same growth factors and cytokines that stimulate liver regeneration which can also stimulate spleen growth. In one study, there was an increase in spleen volume in 78 % of patients following partial hepatectomy for colorectal metastases. There was an average increase in spleen volume of 40–45 % at 6 months after surgery with a decrease in spleen size to near-baseline size at 9 months (Jacobs et al. 2012). There was an inverse correlation between spleen size and platelet count following hepatectomy (Ando et al. 2004).

Splenomegaly is an important cause of thrombocytopenia in hematologic malignancies. Radionuclide scanning reveals differential patterns of splenic cellularity and vascularity in various hematologic disorders. Splenomegaly was attributed primarily due to increased splenic vascularity with polycythemia vera, increased splenic cellularity with chronic myeloid leukemia (CML) and CLL, and both increased splenic vascularity and cellularity in myelofibrosis and hairy cell leukemia (Zhang and Lewis 1989).

Although splenectomy and partial splenic embolization are not commonly performed in patients with solid or hematologic malignancies, such approaches may result in a sustained increase in platelet count and decreased platelet transfusion requirements (Berman et al. 2004; Kauffman et al. 2008; Bhatia et al. 2015).

Thrombocytopenia Due to Cancer Treatment

Systemic Cancer Therapy

Systemic cancer therapy is the most common cause of thrombocytopenia in cancer patients, resulting in thrombocytopenia in nearly two-thirds of patients with solid tumors (Wu et al. 2009). Thrombocytopenia leads to dose reductions in 15 % of cycles and cycle delays in 6 % of cycles in patients receiving chemotherapy for solid tumors. Bleeding is observed in 9 % of patients and is more common in patients with a prior bleeding history, baseline platelet count of less than 75×10^9/l, bone marrow metastases, poor performance status, or with administration of cisplatin, carboplatin, carmustine, or lomustine (Elting et al. 2001). Severity of thrombocytopenia based on National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0 (Table 2) (National Cancer Institute 2010).

Thrombocytopenia with traditional chemotherapy agents is most frequently the result of megakaryocyte cytotoxicity. The typical pattern of thrombocytopenia due to megakaryocyte cytotoxicity is a platelet nadir 7–17 days after drug administration with a return to baseline platelet count in 3–4 weeks. Prolonged thrombocytopenia may be seen with agents that induce bone marrow and stem cell injury, such as busulfan and melphalan (McManus and Weiss 1984). Because of the associated risk of stem cell toxicity and impact on stem cell collection, melphalan is avoided in multiple myeloma patients who are candidates for autologous stem cell transplant.

Thrombocytopenia occurs in over 75 % of patients with colorectal cancer treated with oxaliplatin, 5-fluorouracil, and leucovorin (Andre et al. 2004; Colucci et al. 2005). Although megakaryocyte cytotoxicity is the most commonly recognized mechanism of oxaliplatin-related thrombocytopenia, oxaliplatin has been described to cause thrombocytopenia through splenic sequestration from the sequelae of hepatic sinusoidal injury and immune mediation. Combination therapy with oxaliplatin frequently leads to morphologic changes in the liver, which include sinusoidal obstruction, sinusoidal fibrosis, and veno-occlusive lesions (Rubbia-Brandt et al. 2004). Oxaliplatin-mediated hepatic sinusoidal obstruction has been shown to correlate with increased spleen size and thrombocytopenia. In a retrospective study of 136 patients with early-stage colorectal cancer, there was an increase in spleen size in 86 % of patients receiving adjuvant treatment with 5-fluorouracil and oxaliplatin. There was no change in spleen size in patients who received 5-fluorouracil alone. In nearly a quarter of patients, there was a greater than 50 % increase in spleen size. An increase in spleen size correlated with the cumulative dose of oxaliplatin. There was a significant correlation between the degree of spleen size increase and the reduction in platelet count. Thrombocytopenia due to splenomegaly improved to near-baseline levels 2–3 years after completion of oxaliplatin (Overman et al. 2010).

Table 2 Grading of thrombocytopenia based on National Cancer Institute Common Terminology Criteria for Adverse Events, version 4.0 (National Cancer Institute 2010)

Grade	Platelet level
1	<LLN—75×10^9/l
2	<75–50×10^9/l
3	<50–25×10^9/l
4	<25×10^9/l

LLN lower limit of normal

ITP has also been described with oxaliplatin. Platelet destruction is mediated by oxaliplatin-dependent antibodies to platelet antigens, leading to a sudden drop in platelet count to less than 10×10^9/l and bleeding manifestations within several hours of oxaliplatin infusion (Dold and Mitchell 2003; Curtis et al. 2006). This complication typically occurs after at least 12 cycles of oxaliplatin and may be preceded by hypersensitivity reaction (skin rash, pruritis, chills, and bronchospasm) (Jardim et al. 2012; Bautista et al. 2010). ITP has also been described with fludarabine and irinotecan with differing patterns of total drug exposure and time to platelet nadir following the last dose (Table 3) (Leach et al. 2000; Hegde et al. 2002; Jiang et al. 2014; Bozec et al. 1998; Mirtsching et al. 2014). Alemtuzumab is an anti-CD52 antibody used in the treatment of hematologic malignancies and multiple sclerosis. It is associated with a distinct form of ITP characterized by its delayed onset. In a phase 2 trial of alemtuzumab for the treatment of multiple sclerosis, 6 of 216 (2.8 %) of patients developed ITP. The median time from initial and last alemtuzumab exposure was 24.5 months and 10.5 months, respectively (Cuker et al. 2011). Management of ITP associated with cancer therapy includes discontinuation of implicated drug. Standard treatments of ITP, including steroids and IV immunoglobulin, may be administered to patients with bleeding manifestations or platelets less than 10×10^9/l with favorable outcomes reported.

Thrombotic microangiopathy (TMA) encompasses a spectrum of disorders which are characterized by microvascular thrombosis, hemolytic anemia, platelet consumption, and end-organ damage. While the clinical manifestations overlap, the pathophysiology of TMAs varies based on the underlying etiology. The major subtypes are thrombotic thrombocytopenia purpura and hemolytic uremic syndrome. A variety of secondary causes of TMA have also been described, including infections, collagen vascular disease, malignancies, surgery, stem cell transplant, and drugs.

Nucleoside analogs gemcitabine and mitomycin are the most commonly implicated chemotherapy agents in drug-induced TMA. The estimated incidence of drug-induced TMA is 0.015–0.31 % with gemcitabine and 2–15 % with mitomycin (Fung et al. 1999; Humphreys et al. 2004; Lesesne et al. 1989). Gemcitabine- and mitomycin-induced TMAs are thought to be result of direct endothelial cell injury and occur at higher cumulative doses (Groff et al. 1997; Humphreys et al. 2004; Lesesne et al. 1989). The clinical presentation is characterized by hemolytic anemia, presence of schistocytes, thrombocytopenia, renal insufficiency, elevated LDH, and normal levels of serum metalloproteinase ADAMTS13 (Lesesne et al. 1989; Pisoni et al. 2001; Shah et al. 2013).

Drug-induced TMA has also been reported with several targeted cancer agents, including anti-VEGF agents bevacizumab, sunitinib, and aflibercept (Eremina et al. 2008; Bollee et al. 2009; Izzedine et al. 2007). In a phase I study combining bevacizumab and sunitinib, 5 of 25 patients developed TMA, leading to the abandonment of this combination (Feldman et al. 2009). In contrast to TMA associated with gemcitabine or mitomycin, TMA observed with anti-VEGF agents does not appear to be dose-dependent. Bevacizumab-induced TMA is thought to be the result of inhibition of vascular endothelial growth factor signaling in the kidney (Eremina et al. 2008). In a series of six patients who underwent renal biopsy for evaluation of TMA following bevacizumab, common features included proteinuria and elevated creatinine. However, other manifestations were varied (Eremina et al. 2008).

The optimal management of drug-induced TMA has not been established. Plasma exchange is often employed but is generally not effective. For gemcitabine-induced TMA, the American Society of Apheresis guidelines list plasma exchange as a category IV indication (published evidence demonstrates or suggests apheresis to be ineffective or harmful) based on grade 2C evidence (weak recommendation, low-quality or very low-quality evidence) (Schwartz et al. 2013). Case reports have reported successful treatment with anti-CD20 antibody rituximab for gemcitabine- and mitomycin-induced TMA (Gourley et al. 2010; Shah et al. 2013). Eculizumab is a monoclonal antibody against complement protein C5 which is approved for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. Successful treatment of gemcitabine-induced TMA with eculizumab has also been reported (Al Ustwani et al. 2014).

A variety of other mechanisms of thrombocytopenia have been described with new anticancer agents (Table 4). The onset and duration of thrombocytopenia may be an important indicator of a differing mechanism of thrombocytopenia. In patients with relapsed refractory multiple myeloma

Table 3 Patterns of immune thrombocytopenia with various cancer treatments

Cancer treatment	Exposure	Typical time to onset of thrombocytopenia and/or bleeding from last dose	Platelet nadir	Other associated features
Oxaliplatin	>12 cycles	Within several hours	$<10 \times 10^9$/l	May follow hypersensitivity reaction
Fludarabine	3 cycles	5–12 days	$<10 \times 10^9$/l	–
Irinotecan	4 cycles	Immediately	$<10 \times 10^9$/l	Hemodynamic instability
Alemtuzumab	2.5 cycles	10.5 months	$<10 \times 10^9$/l	–

Table 4 Mechanisms of thrombocytopenia for various cancer treatments and drugs

Mechanism of thrombocytopenia	Anticancer therapy
Stem cell toxicity	Alkylating agents, radiation
Megakaryocyte cytotoxicity	Traditional chemotherapeutic agents
Delayed megakaryocyte maturation	Histone deacetylase inhibitors, trastuzumab emtansine (T-DM1)
Prevention of platelet budding	Bortezomib
Apoptosis	BCL2 inhibitors
Immune-mediated	Fludarabine, irinotecan, oxaliplatin, alemtuzumab
Thrombotic microangiopathy	Mitomycin C, gemcitabine, bevacizumab, sunitinib
Hepatic sinusoidal injury leading to splenomegaly	Oxaliplatin

treated with proteasome inhibitor bortezomib, there is a 60 % decrease in the mean platelet count during treatment followed by rapid recovery between treatments. Mechanistic studies have shown that bortezomib inhibits nuclear factor kappa B, thereby preventing platelet budding from the megakaryocyte (Lonial et al. 2005).

Antiapoptotic BCL-2 family proteins are important for thrombopoiesis and platelet survival. BCL2 inhibitors induce apoptotic death of platelets, leading to a dose-dependent and dose-limiting thrombocytopenia (Vogler et al. 2011; Debrincat et al. 2015). Thrombocytopenia is the most common dose-limiting toxicity of histone deacetylase (HDAC) inhibitors. Mechanistic studies show that HDAC inhibitors delay megakaryocyte maturation through inhibition of GATA binding protein-1 (GATA-1) gene expression in megakaryocytes but do not cause platelet apoptosis (Matsuoka et al. 2007; Bishton et al. 2011). Thrombocytopenia with trastuzumab emtansine (T-DM1) also appears to be due to delayed megakaryocyte differentiation (Uppal et al. 2015).

Radiation Therapy

Acute hematological toxicities are a common finding in patients receiving radiation. The major risk factors for radiation therapy interruptions due to grade 3/4 thrombocytopenia include concurrent chemotherapy (odds ratio [OR] 45.5; $p < 0.001$), increasing percentage of bone marrow irradiation (OR 4.1 for each 20 %; $p < 0.001$), and presence of brain metastases (OR 7.3; $p = 0.01$). Other significant risk factors include leukemia or lymphoma, bone metastases, bone marrow metastases, and prior chemotherapy (Mac Manus et al. 1997). There is an increase in OR of 1.8 for each additional prior line of myelosuppressive chemotherapy (Knox et al. 1997).

Acute hematological toxicities are commonly reported in patients receiving pelvic radiation combined with chemotherapy (Keys et al. 1999; Ajani et al. 2008). A major contributing factor is the large volume of bone marrow irradiated as approximately 50 % of hematopoietically active bone marrow in the body is included within conventional treatment fields (Ellis 1961). Advances in radiation therapy are aimed at maximizing the radiation dose to the tumor while minimizing the radiation exposure to normal tissue, thereby increasing efficacy and decreasing toxicities of treatment. Compared to three-dimensional conformal radiotherapy, intensity-modulated radiation therapy has been showed to decrease volume of bone marrow irradiated, resulting in decreased incidence and severity of acute hematologic toxicity in cervical cancer patients undergoing concurrent chemoradiotherapy (Hui et al. 2014).

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a well-recognized complication of solid and hematologic cancers. DIC is observed in approximately 7 % of patients with solid tumors and approximately 15 % of patients with acute leukemia (Sallah et al. 2001; Dixit et al. 2007). Cancer-associated DIC may be divided into three subtypes based on the predominant pathologic and clinical manifestations: hyper-fibrinolytic, procoagulant, and subclinical (Thachil et al. 2015). The hyper-fibrinolytic form, which is predominantly seen in acute promyelocytic leukemia (APL) and metastatic prostate cancer, is characterized by activation of the fibrinolytic pathway, leading to widespread bruising and bleeding. In APL, early hemorrhagic death from DIC remains an important cause of death in this highly curable disease (Lehmann et al. 2011; Park et al. 2011; McClellan et al. 2012). The procoagulant form of DIC results from excess thrombin generation leading to microvascular and macrovascular thrombosis. It is most commonly seen in adenocarcinomas or pancreatic cancer. Subclinical DIC, which may be seen in a variety of solid tumors, describes DIC diagnosed based on laboratory abnormalities in the absence of clinical manifestations.

In a prospective study of 1117 patients with solid tumors, DIC was found in 76 (6.8 %) patients and was associated with an inferior survival. Risk factors for cancer-associated DIC included older age, male gender, advanced malignancies, breast cancer, and tumor necrosis on pathology specimen. Nearly all patients with cancer-associated DIC presented with platelets less than 150×10^9/l; the median platelet at presentation was 53×10^9/l. Other laboratory findings included prolonged PT in 60 %, prolonged PTT in 53 %,

and fragmented red blood cells in 66 % of patients. Median fibrinogen was 129 mg/dl (Sallah et al. 2001).

The cornerstone of DIC management is to treat the underlying cancer. Further management is based on the clinical manifestations. Patients with bleeding manifestations are treated with platelets, cryoprecipitate, or plasma. Thrombotic manifestations are managed with anticoagulation, preferentially with low molecular heparins.

Immune Thrombocytopenia

ITP is characterized by antibody-mediated peripheral destruction of platelets. Although ITP has been recognized in nearly all lymphoproliferative disorders, it is most commonly described with Hodgkin lymphoma, large granular lymphocyte (LGL) leukemia, and CLL. ITP has been reported in approximately 1 % of patients with Hodgkin lymphoma (Xiros et al. 1988). Platelet count less than 150×10^9/l is observed in 20 % of patients with LGL leukemia. Commonly recognized mechanisms of thrombocytopenia in patients with LGL leukemia include splenomegaly, ITP, and suppression of megakaryopoiesis by LGL cytotoxic lymphocytes (amegakaryocytic thrombocytopenia) (Dhodapkar et al. 1994; Lai et al. 2008).

CLL is characterized by immune dysregulation with common manifestations including autoimmune hemolytic anemia (AIHA), pure red cell aplasia, and ITP (Diehl and Ketchum 1998). ITP is reported in 2–5 % of patients with CLL and its onset may precede, coincide, or follow the diagnosis of CLL (Visco et al. 2008). Compared to CLL patients without ITP, those with ITP have a higher frequency of unmutated immunoglobulin heavy-chain variable region (IgVH) gene (82 % versus 46 %, $p < 0.01$), positive direct antiglobulin test (22 % versus 5 %, $p < 0.001$), AIHA (16 % versus 3 %, $p < 0.001$), and inferior survival (5-year overall survival 64 % versus 82 %, $p = 0.01$). Unmutated IgVH status is a well-recognized negative prognostic marker in patients with CLL and thus is thought be a contributing factor to these survival differences (Visco et al. 2008).

Management

General Management

Patients with solid tumors or hematologic malignancies with "adequate bone marrow function," typically defined as a platelet count of 75–100 $\times 10^9$/l, are generally able to receive full-dose anticancer therapy. Dose delays or reductions are often considered for patients with platelet counts that do not meet this threshold. However, the specific platelet thresholds for dose initiation, delay, or reductions vary based on the anticancer therapy. In contrast, patients with hematologic malignancies involving the bone marrow typically receive full-dose therapy with platelet transfusion support.

According to the American Society of Clinical Oncology clinical practice guidelines, prophylactic platelet transfusion is recommended at a platelet threshold of 10×10^9/l for non-bleeding cancer patients. Higher transfusion thresholds are typically used in patients with active bleeding, high risk for bleeding, fever, hyperleukocytosis, rapidly declining platelet count, or coagulation abnormalities (Schiffer et al. 2001). Antifibrinolytic agents may be used as adjunctive therapy as they have been reported to decrease the number of platelet transfusions, red blood cell transfusions, and bleeding episodes in several small randomized trials of patients with hematologic malignancies (Wardrop et al. 2013).

Role of Thrombopoietin Receptor Agonists in Solid Tumors

Endogenous TPO is a key regulator of megakaryocyte production and maturation (Kuter 2010). TPO receptor agonists, romiplostim and eltrombopag, are widely used in the management of chronic ITP. TPO receptor agonists bind the TPO receptor, thereby activating and stimulating the production of megakaryocytes and inhibiting antibody-mediated apoptosis of megakaryocytes and platelets. There is no evidence to date of TPO receptor expression by tumor cells in preclinical studies, validating the potential safety when used in cancer patients (Erickson-Miller et al. 2010, 2012).

The use of TPO receptor agonist therapy has been explored in several small studies of cancer patients undergoing chemotherapy. In this clinical scenario, the goal of TPO receptor agonist therapy is to avoid platelet nadirs that result in interruptions in treatment or lead to bleeding events without causing thrombocytosis and increased thrombotic risk. The timing and duration of platelet nadir with the specific chemotherapy regimen as well as the time needed for eltrombopag to take effect needs are important considerations in determining appropriate dosing regimen. Pharmacokinetic/pharmacodynamic modeling suggests that eltrombopag administration 5 days before and after chemotherapy results in improved platelet count stability compared to 10 days before or 10 days after chemotherapy (Hayes et al. 2013). This pharmacokinetic/pharmacodynamic modeling should be kept in mind when interpreting clinical trial data exploring TPO receptor agonist therapy in patients receiving chemotherapy.

In a randomized, double-blind, multicenter phase 2 study, patients receiving first-line carboplatin/paclitaxel were randomized to receive 50–100 mg of oral eltrombopag once daily for 10 days following chemotherapy administration. The failure to meet the primary outcome (a difference in platelet count from day 1 in cycle 2 to platelet count in cycle 2 with eltrombopag compared placebo) was likely attributable to suboptimal dosing (Kellum et al. 2010). In a small, blinded, placebo-controlled trial with eltrombopag in patients with solid tumors receiving gemcitabine alone or in combination with cisplatin or carboplatin, patients were randomized to receive oral eltrombopag 100 mg once daily for 5 days before and 5 days after chemotherapy starting with cycle 2. Eltrombopag use was found to be associated with fewer dose reductions or delays (Winer et al. 2015).

Role of Thrombopoietin Receptor Agonists in Hematologic Malignancies

TPO agonists are ineffective in myeloablative states and thus are not used in patients with acute myeloid leukemia (AML) receiving intensive chemotherapy. The administration of pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) has been shown to have no effect on the duration of severe thrombocytopenia or the platelet transfusion requirement in patients receiving myeloablative chemotherapy for AML (Schiffer et al. 2000). The major concern with the use of romiplostim in patients with MDS is the progression to AML based on early trial results (Kantarjian et al. 2010). However, there were similar rates of progression of AML at the time of final analysis (Giagounidis et al. 2014).

Approach to Patients Requiring Anticoagulation for Venous Thromboembolism

Venous thromboembolism (VTE) is a frequent complication of cancer, and importantly, thrombocytopenia does not appear to be protective against the development of VTE (Heit et al. 2000; Vu et al. 2015). The optimal management of cancer-associated VTE in patients with concomitant thrombocytopenia is not well established. Management is largely guided by expert opinion with the aim to balance the risks of recurrent VTE and bleeding during episodes of thrombocytopenia. Low molecular weight heparin (LMWH) therapy remains the standard of care for cancer-associated VTE (Lyman et al. 2015). Vitamin K antagonist therapy should be avoided in patients with severe thrombocytopenia due to its prolonged anticoagulant effect. The International Society on Thrombosis and Haemostasis approach to the management of cancer-associated VTE in patients with thrombocytopenia is presented in Fig. 2 (Carrier et al. 2013). Retrospective data support the safety of this general approach (Babilonia et al. 2014; Miao et al. 2015; Kopolovic et al. 2015). In a retrospective study of 74 cancer patients with a platelet count less than 100×10^9/l at the time of acute VTE diagnosis, 30 patients completed 3 more months at therapeutic LMWH doses with two non-major clinically relevant bleeding events but no major bleeding events. This was in spite of a median platelet nadir of 44×10^9/l (interquartile range: 13–65) and 14 patients having a platelet count nadir of less than 30×10^9/l among this group of 30 patients (Kopolovic et al. 2015). Based on this limited data, full-dose anticoagulation may be considered in selected patients with a platelet count as low as 30×10^9/l. However, well-designed clinical trials are needed

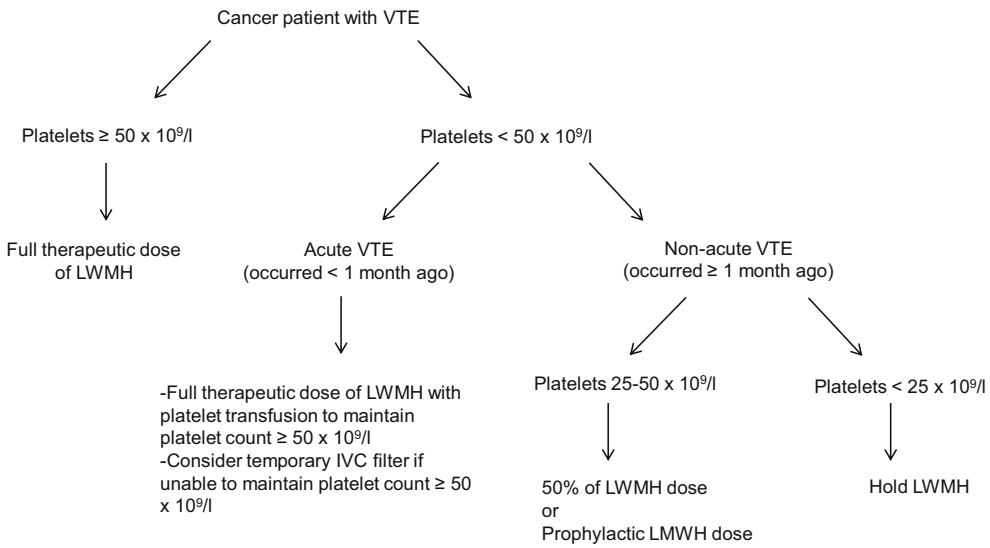

Fig. 2 International Society on Thrombosis and Haemostasis guidelines for the management of venous thromboembolism in cancer patients with concomitant thrombocytopenia (Carrier et al. 2013). *VTE* venous thromboembolism, *LMWH* low molecular weight heparin, *IVC* inferior vena cava

to evaluate the appropriate doses of therapeutic anticoagulation in cancer patients with VTE and concomitant thrombocytopenia.

Take-Home Points

- Thrombocytopenia is a common complication in patients with cancer.
- Systemic cancer treatment is the most common cause of thrombocytopenia in cancer patients.
- The mechanism of thrombocytopenia varies with the cancer treatment.
- Bone marrow involvement with tumor, splenomegaly, disseminated intravascular coagulopathy, and immune thrombocytopenia are other important causes of thrombocytopenia in cancer patients.
- The management of thrombocytopenia in cancer patients includes dose delays or reductions of anticancer therapy and supportive care.

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Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndrome

Johanna A. Kremer Hovinga

Abstract

Thrombocytopenia and microangiopathic hemolytic anemia (MAHA) with schistocytes on the blood smear are key findings in thrombotic microangiopathies (TMAs). Initial clinical evaluation of patients focuses on the distinction between primary and secondary TMA forms associated with underlying diseases or systemic disorders.

Primary TMAs, the focus of this chapter, include thrombotic thrombocytopenic purpura (TTP; acquired or hereditary), Shiga toxin-mediated hemolytic-uremic syndrome (STEC-HUS), and complement-mediated or atypical HUS (hereditary or acquired). Mortality and morbidity resulting from acute TMA episodes are considerable and therefore urgent treatment is necessary, preferably directed at the underlying pathophysiology. Two factors can help to distinguish between the primary TMA forms. Age at presentation is important because HUS is typically diagnosed in children and TTP in (young) adults. Renal involvement may also help because kidney injury is prominent in HUS but usually absent or only moderately present in TTP, where neurological symptoms are more frequent.

A severe ADAMTS13 deficiency (<10 % of that in normal plasma) indicates a diagnosis of TTP. ADAMTS13 deficiency can be autoantibody-mediated (acquired TTP) or the result of *ADAMTS13* gene mutations (hereditary TTP, Upshaw–Schulman syndrome). STEC-HUS is preceded by an infection with Shiga toxin-producing *Escherichia coli* (e.g., *E. coli* O157:H7) or *Shigella dysenteriae* leading to bloody diarrhea. Complement-mediated HUS is the consequence of excessive activation of the alternative pathway of the complement system as a result of mutations in complement regulators or complement factors (hereditary forms) or the presence of autoantibodies against complement regulators (acquired forms).

Plasma exchange (PEX) with fresh frozen plasma replacement is the treatment of choice for TTP, which has the highest acute mortality rate of all TMAs (exceeding 90 % in the pre-plasma therapy era). PEX is usually supplemented by immunosuppressant drugs, corticosteroids, and/or rituximab to tackle ADAMTS13 autoantibodies. In TMA cases with normal or only moderately reduced ADAMTS13 activity, prominent renal involvement, and no history of bloody diarrhea, anti-complement treatment should be considered within the first week of presentation.

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Abbreviations

ADAMTS13	A disintegrin and metalloproteinase with thrombospondin type 1 repeats 13
CI	Confidence interval
ESRD	End-stage renal disease
HELLP	Hemolysis, elevated liver enzymes, low platelet count
HUS	Hemolytic uremic syndrome
MAHA	Microangiopathic hemolytic anemia
OMIM	Online Mendelian Inheritance in Man
PEX	Plasma exchange
STEC-HUS	Shiga toxin-mediated hemolytic uremic syndrome
TMA	Thrombotic microangiopathy
TTP	Thrombotic thrombocytopenic purpura
USS	Upshaw–Schulman syndrome (hereditary TTP)
VWF	Von Willebrand factor

Introduction

Acute thrombotic microangiopathies (TMAs) are life-threatening disorders characterized by microangiopathic hemolytic anemia (MAHA) with schistocytes (Fig. 1) on the peripheral blood smear, consumptive thrombocytopenia, and variable degrees of ischemic organ dysfunction as a result of microvascular thrombosis, primarily affecting the brain and kidneys (Moake 2002; Lämmle et al. 2005; Sadler 2008; Kremer Hovinga and Lämmle 2012; George and Nester 2014). TMAs are rare disorders with an annual incidence of all forms of 11.3×10^{-6} (Terrell et al. 2005).

Depending on the primary organ dysfunction observed, acute TMAs are referred to as thrombotic thrombocytopenic purpura (TTP) when neurological manifestations are predominant, or as hemolytic-uremic syndrome (HUS) in the presence of acute renal insufficiency. Furthermore, distinction can depend on the specialty of the involved consultant, be it a hematologist (TTP) or a nephrologist (HUS), as well as on patient's age, as HUS is mainly found in children, whereas TTP is predominately a disease of (young) adults. The clinical overlap between TTP and HUS is, however, considerable (Amorosi and Ultmann 1966; Rock et al. 1991; Thompson et al. 1992; Vesely et al. 2003).

The primary or idiopathic forms of TTP and HUS must be distinguished from secondary TMAs with underlying or associated disorders, such as drug-induced TMAs, preeclampsia and eclampsia/HELLP syndrome in pregnancy, TMA associated with stem cell transplantation, neoplasia, autoimmune disorders (e.g., systemic lupus erythematosus), and human immunodeficiency virus (HIV) infection (Table 1). These secondary TMA forms are beyond the scope of this chapter. Note, however, that TMA can be associated with severe acquired ADAMTS13 deficiency in patients with systemic lupus erythematosus and that pre-eclampsia often complicates pregnancy in patients with hereditary TTP and congenital ADAMTS13 deficiency (Scully et al. 2006; Fujimura et al. 2009; von Krogh et al. 2014).

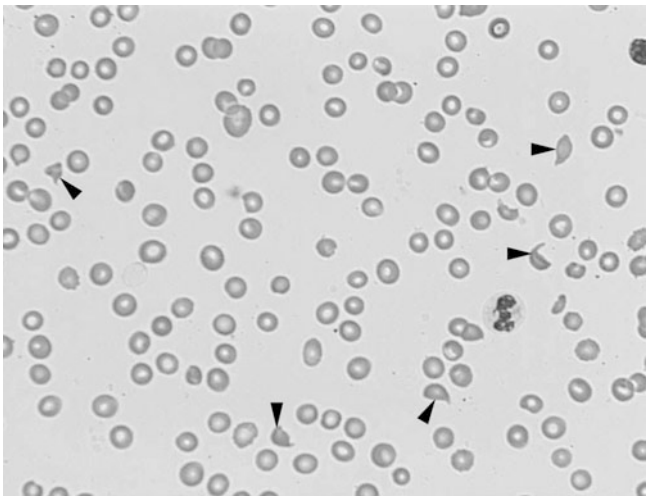

Fig. 1 Peripheral blood smear of a patient presenting with an acute TTP episode. Schistocytes are indicated by *arrowheads*; note the severe thrombocytopenia. Further laboratory work-up revealed the following results: platelet count 8×10^9/L, hemoglobin 46 g/L, LDH 2655 U/L (normal range <480 U/L), total bilirubin 105 μmol/L (3–26 μmol/L), creatinine 91 μmol/L (59–116 μmol/L). The direct antiglobulin test (direct Coombs test) was negative. ADAMTS13 activity was <5 % in the presence of an ADAMTS13 inhibitor of 3 BU/mL, proving a diagnosis of acute acquired TTP as a result of severe autoantibody-mediated ADAMTS13 deficiency. The patient was treated with daily plasma exchange and prednisone, and was splenectomized on day 29 as a result of a plasma refractory state. He has remained in remission, despite the reappearance of a severe functional ADAMTS13 inhibitor 3.4 years after splenectomy (Kremer Hovinga et al. 2004b)

During the past decade, a number of pathophysiological processes underlying TTP and HUS have been unraveled. This has provided the rationale for long-standing, empirical therapies but also directed the development of new treatment strategies such as rituximab for acquired TTP, development of recombinant ADAMTS13 for hereditary TTP, and anti-complement therapy in atypical HUS (aHUS).

This chapter focuses on the clinical presentation, pathophysiological findings, and treatment options for TTP and HUS.

Epidemiology

Thrombotic Thrombocytopenic Purpura

Annual incidence rates for idiopathic TTP were estimated to be $3.7–4.43 \times 10^{-6}$ (Török et al. 1995; Terrell et al. 2005) and that of acquired TTP with severe ADAMTS13 deficiency caused by anti-ADAMTS13 autoantibodies as

Table 1 Forms of thrombotic microangiopathy with underlying pathophysiology and primary management options

Primary forms of thrombotic microangiopathy		
	Cause	Treatment
Thrombotic thrombocytopenic purpura (TTP)		
Hereditary TTP (Upshaw–Schulman syndrome)	Congenital ADAMTS13 deficiency Homozygous or compound heterozygous *ADAMTS13* gene mutations	Plasma infusion Recombinant ADAMTS13 in the future
immune-mediated, acquired TTP	Acquired ADAMTS13 deficiency as a result of inhibitory and noninhibitory anti-ADAMTS13 autoantibodies In roughly one-third of patients, ADAMTS13 activity is normal or only mildly reduced	Plasma exchange with replacement of plasma Immunosuppression (corticosteroids, second line rituximab, splenectomy)
Hemolytic uremic syndrome (HUS)		
Shiga toxin-mediated HUS	Infection, mainly enteric, with Shiga toxin-producing bacteria, mainly *E. coli* (predominately serotype O157:H7 followed by O26, O145;O128, etc.) and *S. dysenteriae type 1*	Supportive care
Post-pneumococcal infection HUS	Pneumococcal neuraminidase exposes usually cryptic Thomsen–Friedenreich antigen on red blood cells and glomeruli	Supportive care
Atypical HUS	Mutations in complement regulators CFH (20–30 %), CFI (5–10 %), MCP (5–10 %), THBD (3–5 %) or in complement factors (gain-of-function) C3 (5–10 %) and CFB (1–4 %) Mutations in DGKE (rare)	Plasma infusion or exchange Anti-C5 antibody or other anti-complement treatment
Atypical HUS	Anti-complement factor H antibodies in homozygous deletion of CFHR 1/3	Plasma infusion or exchange Immunosuppression Anti-C5 antibody or other anti-complement treatment
Secondary forms of thrombotic microangiopathy (TMA)		
	Cause	Treatment
Pre-eclampsia and eclampsia/HELLP syndrome	Many theories Typical pregnancy complication in many female hereditary TTP patients Mutations in complement factors and regulators as risk factors in some patients/families	Control of blood pressure and urgent delivery Early preventive administration of plasma
Drug-mediated TMA	Quinine (direct via quinine-dependent antibodies) Ticlopidin (in some by induction of anti-ADAMTS13 antibodies) VEGF inhibition Gemcitabine, others	Cessation of drug Supportive care
Connective-tissue disease-associated TMA	Systemic lupus erythematosus and others About half of cases overlap with TTP and severe acquired ADAMTS13 deficiency Frequently noninhibitory anti-ADAMTS13 antibodies	Treatment of underlying illness Plasma exchange Immunosuppression
Hematopoietic stem cell transplantation-associated TMA	Calcineurin inhibitors Graft-versus-host disease towards endothelium? Dysregulation of the complement system?	Mild forms: Calcineurin inhibitors Supportive care Severe forms: Grim prognosis Anti-C5 antibody or other anti-complement treatment
Neoplasia-associated TMA	Advanced carcinoma (typically of stomach, breast, or prostate)	Chemotherapy may be tried, prognosis generally grim Supportive care
HIV-associated TMA	Ongoing uncontrolled HIV infection In some cases classical TTP with severe acquired ADAMTS13 deficiency	Highly active antiretroviral therapy (HAART) Plasma exchange

2.17×10^{-6} (95 % CI 2.00–2.34) (Reese et al. 2013). The latter study confirmed the clinical observation that acquired TTP is rare in children under 18 years of age (annual incidence rate 0.09×10^{-6} compared with 2.88×10^{-6} in adults) as well as the observation that blacks are more often affected than non-blacks (ratio 7.1:1), as are women (female to male ratio 3.2:1) (Reese et al. 2013).

The true prevalence of hereditary TTP as a result of congenital ADAMTS13 deficiency, also known as Upshaw–Schulman syndrome (USS, OMIM #274150), is unknown. A proportion of <5 % of all TTP cases with severe ADAMTS13 deficiency has been put forward (Galbusera et al. 2009). Worldwide, more than 150 affected patients are known to be alive. A population screening in healthy

Japanese adults, stratified according to their plasma ADAMTS13 activity, revealed a considerable number of heterozygous *ADAMTS13* mutation carriers in this population (Kokame et al. 2011). The authors hypothesized that the true prevalence of hereditary TTP could be up to threefold higher than the estimate based on recognized patients. The point prevalence of hereditary TTP in central Norway, a region with many USS patients, was 16.7×10^{-6} (von Krogh et al. 2016). This number was reflected by an allelic frequency of the two most prevalent *ADAMTS13* mutations in the population, *c.4143_4144dupA* and p.R1060W, of 0.04–0.33 %, and 0.3–1 %, respectively. For the latter mutation, a somewhat lower allelic frequency of 0.06 % was documented in a population study in the Netherlands (de Vries et al. 2015).

Hemolytic Uremic Syndrome

HUS is predominately observed in children below 5 years of age. More than 90 % of cases develop secondary to infections with Shiga toxin (Stx)-producing *E. coli* (STEC), *Shigella dysenteria* and *Streptococcus pneumonia* (Tarr 2009; Noris et al. 2012). For different countries in Europe and the Americas, annual incidence rates of infectious HUS of 2×10^{-6} to 7.1×10^{-6} children per year have been reported (Gianviti et al. 1994; Decludt et al. 2000; Banatvala and Zwi 2000; Elliott et al. 2001; Gerber et al. 2002; Schifferli et al. 2010). In the majority of cases, these infections are mild and self-limiting; however, about 10–15 % of affected patients subsequently develop STEC-HUS (Wong et al. 2000; Tarr et al. 2005).

Apart from large outbreaks, often attracting public attention (Buchholz et al. 2011; Frank et al. 2011), most occurrences are sporadic. In Switzerland, infections with Stx-producing bacteria are reportable, with roughly 200 cases reported every year (the resident population of Switzerland is eight million). About 10–20 % of all individuals, but up to 80 % of children below the age of 5 years (Tarr 2009), develop a severe course with bloody diarrhea and hemorrhagic colitis. Of these, 10–15 % subsequently develop STEC-HUS (Wong et al. 2000; Tarr et al. 2005). Consequently, STEC-HUS is also referred to as diarrhea-associated (D^+HUS) or typical HUS.

The most common bacteria implicated in STEC-HUS is *E. coli* serotype O157:H7, which accounts for up to 70 % of cases in North America and western Europe (Ochoa and Cleary 2003; Barton Behravesh et al. 2011; Scallan et al. 2011; Locking et al. 2011; Gould et al. 2013). Several non-O157:H7 strains (i.e., O111:H8, O103:H2, O121, O145, O113, or O104:H4, in the German 2011 outbreak) were also implicated (Gerber et al. 2002; Bundesamt and Gesundheit 2008; Buchholz et al. 2011; Trachtman 2012). Infections with *Shigella dysenteriae* type 1 are frequently identified in STEC-HUS in developing countries. Healthy cattle are the major reservoir of *E. coli* O157:H7 and, consequently, consumption of contaminated meat or unpasteurized dairy products are among the most common modes of food-borne transmission.

About 10 % of HUS cases are classified as atypical because they are not caused by Stx-producing bacteria or *Streptococcus pneumonia*. Mutations in genes encoding complement system proteins [gain-of-function mutations in complement factor genes *C3* and *CFB*, as well as loss-of-function mutations in complement regulatory protein genes *CFH*, *CFI*, *MCP* (also known as *CD46*), or in the thrombomodulin gene *THBD*] are identified in 50–70 % of aHUS and have been found in sporadic and familial cases (Noris and Remuzzi 2009; George and Nester 2014). Rare cases have been attributed to acquired complement dysregulation caused by antibodies to CFH and CFB (Noris and Remuzzi 2009).

Pathophysiology

Thrombotic Thrombocytopenic Purpura

The pathophysiological hallmark of acute TTP is occluded microvasculature caused by von Willebrand factor (VWF)–platelet-rich thrombi. These thrombi are the consequence of insufficient processing of newly secreted, unusually large, and extremely adhesive VWF multimers (Sadler 2006). In a substantial number of patients, a severe deficiency of the von VWF-cleaving protease, also known as ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motifs-13), prevents physiological processing of unusually large VWF multimers into smaller, less-adhesive and sticky VWF molecules.

At present, two forms of severe ADAMTS13 deficiency (<10 % of normal activity) are distinguished. In USS (Upshaw 1978; Schulman et al. 1960) or hereditary TTP, severe ADAMTS13 deficiency is the result of compound heterozygous or homozygous mutations in the *ADAMTS13* gene (Levy et al. 2001; Kokame et al. 2002, 2008; Schneppenheim et al. 2003, 2006; Assink et al. 2003; Matsumoto et al. 2004a; Veyradier et al. 2004; Donadelli et al. 2006). Obligatory heterozygous *ADAMTS13* mutation carriers typically have an ADAMTS13 activity that is about 50 % of the normal and are generally considered to be healthy, although mild thrombocytopenia during pregnancy or infections has been reported in some cases (Kremer Hovinga and Meyer 2008; Mansouri Taleghani et al. 2013).

The *ADAMTS13* gene is located on chromosome 9q34, in close neighborhood to the *ABH blood group locus*, and encodes a multidomain protein of 1427 amino acids. More than 100 different causative *ADAMTS13* mutations,

including missense (~62 %), nonsense (~12.5 %), splice site (~8 %), and frame-shift (deletions and insertions; 17.5 %) mutations, spreading over all ADAMTS13 protein domains, have been identified in USS. The majority of mutations are confined to single families; consequently, homozygous mutation carriers are mainly observed in offspring of consanguineous marriages. Exceptions are two recurring mutations, *c.4143_4144dupA* in exon 29 and p.R1060W in exon 24, which have been observed in several unrelated families (Schneppenheim et al. 2003, 2006; Assink et al. 2003; Pimanda et al. 2004; Tao et al. 2006; Donadelli et al. 2006; Hrachovinova et al. 2006; Manea et al. 2007; Garagiola et al. 2008; Camilleri et al. 2008) and have a widespread geographical distribution. The frameshift mutation *c.4143_4144dupA* has been described in families originating from central and northern Europe, and was found to account for many cases in Scandinavia and around the Baltic Sea (Schneppenheim et al. 2006; Hyla-Klekot et al. 2013; von Krogh et al. 2016). Distribution of the second mutation, *c.3178 C>T* (p.R1060W), is even more extended and is the predominant mutation in British patients, but has also been reported in patients from Turkey, central Europe, Scandinavia, France, and Italy, as well as in North Americans of European descent (Tao et al. 2006; Camilleri et al. 2008; Lotta et al. 2010). The mutation was also found in the North American population of the NHLBI GO Exome Sequencing Project and the CEPH sample in the 1000 Genomes Browser (listed as single nucleotide polymorphism rs142572218).

The clinical phenotype of hereditary TTP is variable. There seems to be a dichotomous distribution of disease onset with a number of patients presenting with a first acute TTP episode before the age of 5 years (early onset), whereas others remain asymptomatic into early adulthood and suffer from the first acute episode between 20 and 40 years of age or later (late onset) (Furlan and Lämmle 2001), often triggered by a first pregnancy (Fujimura et al. 2009; Moatti-Cohen et al. 2012; Scully et al. 2014; von Krogh et al. 2014). In analogy to hemophilia, where patients with levels of blood-clotting protein FVIII:C of less than1 % usually have a severe, those with 1–5 % a moderate, and patients with >5 % a mild disease course, it was hypothesized that the severity of the clinical phenotype (defined by the timepoint of the first acute TTP episode, frequency of recurrence, and need for prophylactic plasma infusion) is controlled by the presence and amount of residual ADAMTS13 activity (Meyer et al. 2008b; Lotta et al. 2012). Residual ADAMTS13 activity was essentially found in carriers of the *c.3178 C>T* (p.R1060W) mutation, with the highest values in p.R1060W homozygotes. In these patients ADAMTS13 activities ≥10 % were associated with an asymptomatic course up to the seventh decade (Meyer et al. 2008b). A simple *ADAMTS13* genotype–phenotype correlation is, however, questioned by the variable clinical courses of homozygous *c.4143_4144dupA* patients, even within families (Schneppenheim et al. 2006; von Krogh et al. 2016).

It is intriguing to speculate on modifier genes, especially in the complement system, given the overlapping clinical pictures of TTP and HUS. Noris et al. (2005) reported on such an additional *CFH* mutation, previously described in aHUS in a hereditary TTP patient with primarily renal involvement. The *CFH* mutation was lacking in other family members with congenital ADAMTS13 deficiency having no renal involvement. In a recent study by Fan et al. (2016) on 32 hereditary TTP patients, of whom 13 had (severe) renal involvement up to end-stage renal disease (ESRD) and kidney transplantation, the missense sequence variants (single nucleotide polymorphisms) in complement factor and regulatory genes, reported to be associated with an increased risk for aHUS, were not more common in USS patients with renal insufficiency compared with USS patients without. However, 1/13 patients with renal involvement had a novel C3 mutation, p.K155Q located in the C3 macroglobulin-like 2 domain, where other predisposing aHUS mutations cluster. Although complement aberrations could contribute to renal involvement, there have to be other, as yet unrecognized, factors shaping the phenotype in congenital ADAMTS13 deficiency.

Acquired TTP is an autoimmune disorder with circulating anti-ADAMTS13 autoantibodies, leading to a severe ADAMTS13 deficiency. Initial retrospective studies found a severe ADAMTS13 deficiency in 87–100 % of patients clinically diagnosed with idiopathic (or classical) TTP (Furlan et al. 1998a; Tsai and Lian 1998). Subsequent studies on less-selected patient cohorts, however, reported lower frequencies of between 33 and 80 % [for a review see (Kremer Hovinga and Lämmle 2012)] with a pooled average of ~60 % (Furlan et al. 1998a; Tsai and Lian 1998; Veyradier et al. 2001; Vesely et al. 2003; Matsumoto et al. 2004b; Kremer Hovinga et al. 2004a; Zheng et al. 2004; Peyvandi et al. 2004; Böhm et al. 2005; Scully et al. 2008). There has been ample debate on the reasons for this variable correlation between severe ADAMTS13 deficiency and clinical diagnosis of acute TTP. Possible explanations are differences in the assays used for ADAMTS13 activity determination, single versus multicenter studies using different diagnostic criteria, and variable attention to underlying secondary causes of TMA such as TMA after stem cell transplantation or associated with drugs or advanced cancers, where ADAMTS13 activity is usually normal or only mildly reduced (Kremer Hovinga et al. 2010; Peyvandi et al. 2004, 2008; Vesely et al. 2003; Veyradier et al. 2001; Zheng et al. 2004).

The prerequisite of a positive antibody assay in the context of severe ADAMTS13 deficiency (whether functional

inhibitor assay or ELISA) is the presence of free, circulating anti-ADAMTS13 antibodies. In the majority of patients, strong functional ADAMTS13 inhibitors can be demonstrated by Bethesda-like assays (Furlan et al. 1998a, b; Tsai and Lian 1998). Some 10-25 % of cases have only noninhibitory anti-ADAMTS13 antibodies, which are also present in 4–15 % of healthy blood donors and in a number of hereditary TTP patients on regular plasma prophylaxis; however, in the latter these antibodies do not seem to interfere with ADAMTS13 recovery or ADAMTS13 plasma half-life (Fujimura et al. 2011; Kremer Hovinga and Meyer 2008). Noninhibitory anti-ADAMTS13 antibodies have been postulated to act through ADAMTS13 clearance (Scheiflinger et al. 2003; Rieger et al. 2005, 2006; Thomas et al. 2015). Because ADAMTS13 antigen values are severely reduced in the majority of acquired TTP patients, the latter mechanism is probably important for all types of anti-ADAMTS13 antibodies (Rieger et al. 2006; Feys et al. 2006; Thomas et al. 2015). Unexpectedly high ADAMTS13 antigen levels in samples from patients with severe ADAMTS13 deficiency in the presence of anti-ADAMTS13 antibodies prompted the measurement of circulating immune complexes of anti-ADAMTS13 antibody and ADAMTS13 (Rieger et al. 2006). These were subsequently found to be a dominant aspect of acquired TTP and present during acute episodes and relapses but also in remission, even years after the previous acute TTP episode (Froehlich-Zahnd et al. 2012; Ferrari et al. 2012, 2014).

Anti-ADAMTS13 antibodies are mainly IgG isotypes and predominantly of subclass IgG_4, followed by subclasses IgG_1, IgG_2, and IgG_3 (Ferrari et al. 2009). High levels of IgG_4 were found to be associated with an increased risk of relapse and, in relapsed cases, IgG_4 was often the only anti-ADAMTS13 isotype. On the other hand, the presence of IgA and/or IgG_1 at presentation with acute TTP was associated with adverse outcome in a small number of patients (Ferrari et al. 2007, 2009).

ADAMTS13 autoantibodies frequently use the VH1-69 and VH1-3 heavy chain gene segments (Pos et al. 2009; Schaller et al. 2014). Somatic mutation rates of characterized monoclonal anti-ADAMTS13 antibodies are compatible with affinity maturation (Schaller et al. 2014; Luken et al. 2006; Pos et al. 2009). In longitudinal studies of patients over a number of relapses, functional maturation (from noninhibitory to inhibitory anti-ADAMTS13 antibodies) and/or epitope spreading was also demonstrated, suggesting continuous development and shaping of the autoimmune response to ADAMTS13 in acquired TTP (Froehlich-Zahnd et al. 2012; Thomas et al. 2015).

The principal epitope of anti-ADAMTS13 antibodies lies within the ADAMTS13 spacer domain and was fine-mapped to positively charged amino acids Arg568 and Arg660, as well as to Phe592, Tyr661, and Tyr665 (Jin et al. 2010; Pos et al. 2010, 2011). Anti-spacer domain antibodies are present in 97–100 % of patients with severe acquired ADAMTS13 deficiency (Klaus et al. 2004; Luken et al. 2005; Zheng et al. 2010; Thomas et al. 2015), and inhibitory anti-ADAMTS13 antibodies were only found among anti-spacer domain antibodies (Thomas et al. 2015). Roughly two-thirds of patients also have antibodies that react with other ADAMTS13 domains. So far, epitopes of these latter antibodies have not been fine-mapped.

As in other autoimmune diseases, there is evidence for a certain genetic predisposition for the development of pathogenic ADAMTS13 antibodies. The major histocompatibility complex (MHC) class II allele HLA DRB1*11 was found to be over-represented in acquired TTP patients in different European countries, whereas the HLA DRB1*04 allele was more prevalent among healthy controls, suggesting a protective effect of the latter MHC class II allele (Coppo et al. 2010a; Scully et al. 2010). Furthermore, a pair of identical twin sisters, not having the HLA DRB1*11 allele, developed acute TTP as a result of severe autoantibody-mediated ADAMTS13 deficiency a little over a year apart (Studt et al. 2004). Heterozygous *ADAMTS13* mutation carriers have been observed among patients diagnosed with acute TTP caused by severe acquired ADAMTS13 deficiency (in 11 % and 9.6 % of the studied acquired TTP populations, respectively) (Meyer et al. 2007; Camilleri et al. 2008). Seven of these heterozygous ADAMTS13 mutation carriers had ADAMTS13 IgG antibodies, which were found to be functional ADAMTS13 inhibitors in six of the seven patients. Given the low prevalence of *ADAMTS13* mutations in the general population, the number of heterozygous mutation carriers among patients diagnosed with acquired TTP seems remarkable and hints to a role of heterozygous sequence variants either in facilitating a drop in ADAMTS13 to below a critical threshold, relevant to set off an acute TTP episode, or in the development of ADAMTS13 autoantibodies.

Hemolytic Uremic Syndrome

Pathological-anatomical findings in STEC-HUS and aHUS are thickening of arterioles and capillary walls as a result of endothelial swelling and detachment, accumulation of subendothelial cell debris and protein deposits, and narrowing of vessel lumens, fortified by obstructing platelet thrombi.

Enterohemorrhagic bacteria release Shiga toxins (Stxs) into the gut, from where they are absorbed across the epithelium into the blood stream. They bind to endothelial cells, predominately in the small vessels of gut, kidney, and brain, leading to bloody diarrhea, renal insufficiency, and neurological complications (Bielaszewska and Karch 2005; Karch

et al. 2006). Comparison of *Stx* genotypes in isolates from patients with uncomplicated STEC diarrhea and from patients who developed STEC-HUS associated *Stx2* with severe disease course [for a review see (Karch et al. 2006)].

Shiga toxins are the major virulence factor of enterohemorrhagic bacteria and belong immunologically to two major families, Stx1 and Stx2. In *E. coli*. Stx1 is 56 % homologous to Stx2 and differs only by one amino acid from *S. dysenteriae* type 1 Stx. Besides the major toxin types Stx1 and Stx2, a number of sequence variants, particularly for *Stx2* (e.g., *stx2a*, *stx2c*, and *stx2d*) have been described (Karch et al. 2006). Stxs are composed of five glycolipid-binding B subunits, which facilitate binding to globotriaosylceramide (Gb3) on epithelial and endothelial cells, and one enzymatically active A subunit that inhibits protein synthesis by cleaving ribosomal RNA, eventually leading to apoptotic cell death. Besides Stxs, there are a number of other virulence factors that contribute to the pathogenicity of STEC, such as intimin (encoded by *E. coli* attaching and effacing gene, *eae*), an adhesin (encoded by *saa*), or enterohemolysin (encoded by *ehxA*). Their roles in human disease, however, are still under investigation.

The complement system is part of the innate immune system. The C3 and C5 convertase complexes are formed via three pathways: classical, lectin and alternative pathways, leading finally to the generation of the lytic membrane attack complex C5b-9. Host cells are normally protected by membrane-anchored or fluid-phase complement regulators that inactivate and dissociate the C3 and C5 convertases (George and Nester 2014; Nester and Thomas 2012; Noris and Remuzzi 2009). Atypical HUS is linked to uncontrolled activation of the complement system, either as a result of gain-of-function mutations in C3 or *CFB*, or through insufficient complement regulation. Most prevalent are *CFH* mutations, accounting for roughly 25 % of identified mutations in aHUS, followed by mutations in membrane cofactor protein *MCP* (5–10 %), *CFI* (5–10 %), *C3* (5–10 %), and *CFB* (1–4 %) (George and Nester 2014; Nester and Thomas 2012; Noris and Remuzzi 2009). In addition, components of coagulation and other pathways can modulate complement activation. Sequence variants or mutations in the thrombomodulin gene (*THBD*, 3–5 %) (Delvaeye et al. 2009), the plasminogen gene (*PLG*) (Bu et al. 2014), or the diacylglycerol kinase-ε gene (*DGKE*) (Lemaire et al. 2013) have been implicated in small numbers of aHUS patients. In addition, homozygous deletions of *CFH-related proteins 1* and *3* are associated with autoantibody formation against CFH and are identified in about 6 % of aHUS patients.

Mutations are often heterozygous, and phenotypic penetrance among carriers of *CFH, CFI*, *MCP*, and *THBD* mutations have been reported to be 40–50 % (Caprioli et al. 2006; Delvaeye et al. 2009). It was suggested that mutations in complement system genes are risk factors rather than causes of aHUS. In many patients, additional genetic risk factors have been identified, suggesting that more than one risk factor is required for overt phenotypic manifestation in aHUS. Overall complement regulation can be maintained in the presence of reduced action of one or two regulators; however, situations associated with complement activation and inflammation, such as infection or pregnancy, confer additional risk factors and precede acute syndromic presentation in up to half of mutation carriers (Caprioli et al. 2006; Delvaeye et al. 2009).

Clinical Presentation and Diagnosis

In 1966, Amorosi and Ultman reviewed their own 16 TTP cases as well as cases reported in the literature. They summarized the most prevalent clinical signs into the famous TTP pentade of MAHA, thrombocytopenia, neurological manifestations, renal insufficiency, and fever (Table 2). There were no established therapies and mortality exceed 90 %. Nowadays, with good treatment options available and high mortality in untreated patients, acute TTP can be diagnosed on the basis of the concomitant presence of (severe) thrombocytopenia and MAHA with schistocytes on the blood smear, without another apparent explanation (George 2000, 2010). Only a minority of patients presents with the full pentade of signs and organ manifestations nowadays. However, myocardial ischemia and ischemia of intestinal organs are increasingly recognized in patients and can influence management (e.g., hospitalization in intensive care rather than a normal ward when cardiac involvement is present) (Sane et al. 2009; Benhamou et al. 2015; Hughes et al. 2009; McDonald et al. 2009; Swisher et al. 2007).

Basic laboratory work-up should include a full blood count with differential markers of hemolysis (reticulocyte count, haptoglobin, lactate dehydrogenase, bilirubin, and direct Coombs test, which should be negative) and of organ involvement (cardiac troponin, creatinine, possibly S100b as neurological marker). Prior to the first administration of plasma-containing blood products, a blood sample should be withdrawn for ADAMTS13 activity measurement. At the beginning of the millennium, ADAMTS13 assays were cumbersome and confined to specialized research laboratories, today a wide range of commercial assays, which are generally easy to perform and have short turnaround times, are available and results are usually on hand within a few days. An ADAMTS13 activity of <10 % of the normal confirms a diagnosis of acute TTP. The presence of a functional ADAMTS13 inhibitor and/or of anti-ADAMTS13 antibodies allows the distinction between acquired and congenital severe ADAMTS13 deficiency. Roughly one third of patients clinically diagnosed with acute TTP in the absence of an apparent

Table 2 Clinical presentation and survival of acute TTP/TMA over the past nine decades

	Amorosi and Ultman (1966)	Ridolfi and Bell (1981)	Thompson et al. (1992)	Rock et al. (1991)	Vesely et al. (2003)	Kremer Hovinga et al. (2010)	
Study type	Retrospective and literature	Retrospective and literature	Retrospective	Prospective	Prospective	Prospective	
						ADAMTS13 >10 %	ADAMTS13 <10 %
Observation period	1925–1962	1964–1980	1980–1991	1982–1989	1989–2001	1995–2008	
Number of cases	271[a]	275[a]	40	102	142	201	60
Thrombocytopenia (%)	96	96	100	100	100	100	100
MAHA (%)	96	98	100	100	100	100	100
Neurological dysfunction (%)	92	84	70	63	50	44	50
Renal abnormalities (%)	88[b]	76[b]	54[b]	59[b]	43[c]	54[c]	10[c]
Fever (%)	98	59	60	24	Not reported	Not reported	Not reported
Survival (%)	10	46	80	PEX 78 PI 50	All 50 iTTP 69	68	78

MAHA microangiopathic hemolytic anemia, *PEX* plasma exchange, *PI* plasma infusion, *iTTP* idiopathic TTP
[a]Number of studied cases was not the same for all reported variables
[b]Renal abnormalities not defined, only yes/no answer
[c]Acute renal failure

underlying disorder have normal or only mildly to moderately reduced ADAMTS13 activity. In a number of the latter patients, usually with a good response to plasma exchange (PEX) treatment, the presence of anti-ADAMTS13 antibodies was demonstrated. These even evolved into functional ADAMTS13 inhibitors over subsequent TTP episodes in one patient (Froehlich-Zahnd et al. 2012; Kremer Hovinga and Lämmle 2012). This suggests that ADAMTS13 may be specifically involved in the disease in the absence of a severe ADAMTS13 deficiency, as assessed by current routine assays.

Patients with a severe acquired ADAMTS13 deficiency are a distinct group of patients that usually have a lower platelet count, less severe or no renal involvement, and a high risk of relapse in survivors (Kremer Hovinga et al. 2010; Coppo et al. 2010b).

Hereditary TTP can present in early childhood or later in life. Concise exploration of case histories revealed that many patients had exchange transfusions within the first days of life [28 % of Japanese patients (Fujimura et al. 2011, 2009) and 44 % of Norwegian patients (von Krogh et al. 2016)]. During childhood, episodes of (mild) thrombocytopenia and/or hemolytic anemia are frequently documented in the context of mild infections (Schneppenheim et al. 2003). Later in life, pregnancy is an important risk factor for a first acute TTP episode (typical constellation of late disease onset). This is particularly true for carriers of the *ADAMTS13* p.R1060W mutation, where acute TTP episodes are rarely seen outside of situations with increased risk (Lotta et al. 2012; Scully et al. 2014; Moatti-Cohen et al. 2012). Nearly all women with hereditary TTP develop pregnancy complications; thrombocytopenia is usually present during the second and third trimester, and pre-eclampsia and eclampsia/HELLP are frequently observed.

A diagnosis of hereditary TTP is based, according to the criteria of the hereditary TTP registry (www.ttpregistry.net, ClinicalTrials.gov identifier NCT01257269), on a suitable patient and/or family history with $\geq$1 acute TTP episodes; an ADAMTS13 activity of <10 % of the normal in the absence of a functional ADAMTS13 inhibitor on two or more occasions at least 1 month apart; molecular analysis of the *ADAMTS13* gene revealing bi-allelic *ADAMTS13* mutations; and/or a plasma infusion trial documenting full recovery and a plasma half-life of infused ADAMTS13 of 2–4 days (Furlan et al. 1999; Meyer et al. 2008a).

The diagnosis of infection-associated HUS is based on the patient's age [typically <5 years of age (Wong et al. 2000) but can also be observed in adults (Dundas et al. 1999)], a history of prodromal diarrhea that turns bloody after 1–2 days, and an overt clinical triade of thrombocytopenia, MAHA, and often severe renal insufficiency that becomes apparent after 5–7 days. Serotype-specific enzyme immunoassays are available for the detection of *E. coli* O157

directly from stool samples. Nowadays, PCR assays for *Stxs* and multiplex PCR assays for other virulence factors (e.g., intimin and enterohemolysin) on stool specimens are standard practice.

About two-thirds of patients require dialysis during the acute phase (Decludt et al. 2000; Banatvala and Zwi 2000; Elliott et al. 2001; Gerber et al. 2002) and, although renal function recovers in the majority of survivors, an average of 25 % (range 0–64 %) (Garg et al. 2003) demonstrate long-term renal sequelae (such as reduced glomerular filtration rate, hypertension, or proteinuria) and 3 % present with end-stage renal disease (ESRD) (range 0–17 %) (Garg et al. 2003) requiring long-term renal replacement therapy. Neurological symptoms, including stroke, seizure, and coma, are present in 25–30 % of cases. Mortality rates during the acute phase of STEC-HUS have been reported to be 3–5 % and death is nearly always associated with extrarenal disease, particularly severe neurological involvement (Tonshoff et al. 1994; Siegler and Oakes 2005; Garg et al. 2003).

The distinction between acquired TTP and aHUS is more difficult, as no objective single test is available to confirm the clinical diagnosis of aHUS. A diagnosis is established in the presence of MAHA, thrombocytopenia, serum creatinine level >200 μmol/L (>2.25 mg/dL), negative stool examination for Stx-producing bacteria, and ADAMTS13 activity of >10 %. Molecular analysis of complement factor genes are now available from a number of commercial and research labs; however, documentation of such mutations is not necessary to establish a diagnosis of aHUS. Until recently, prognosis of aHUS has been grim with a mortality rate of 25 % during the acute phase, and ESRD or serious neurological sequelae in up to 50 % of surviving patients (Taylor et al. 2004; Schieppati et al. 1992; Constantinescu et al. 2004).

Treatment and Outcome

For clarity, uniform criteria for patient outcomes are required. The following definitions are in accordance with the Oklahoma TTP-HUS registry (Kremer Hovinga et al. 2010; Vesely et al. 2003) and are widely used today to assess treatment in TTP:

- Response to treatment is defined as achievement of a normal platelet count of $\geq 150 \times 10^9$/L and normalization of lactate dehydrogenase (LDH) values during PEX.
- Remission is defined as no PEX for 30 days, which is usually associated with resolution of laboratory and clinical abnormalities.
- Exacerbation is defined as recurrence of thrombocytopenia and/or MAHA within 30 days of the last PEX session, necessitating resumption of daily PEX.
- Relapse (i.e., a new episode) is defined as recurrence of thrombocytopenia and/or MAHA after achievement of remission.

When Amorosi and Ultman described the famous pentade of TTP in 1966, the mortality in their series of more than 250 patients exceeded 90 %. For patients treated with corticosteroids, mortality was 77.5 % (9/40 patients survived), and 65 % among patients splenectomized early.

Plasma Therapy

In the late 1970s, Bukowski et al. (1977), Byrnes and Khurana (1977), and Upshaw (1978) first reported successful treatment of acute TTP episodes by PEX with replacement of plasma and by plasma infusion. Thereby, they demonstrated that an important aspect of TTP treatment is replacement of a missing plasma factor.

Between 1982 and 1989, the Canadian Apheresis Study Group performed the first randomized trial on 102 patients presenting with acute TTP, comparing PEX with replacement of fresh frozen plasma (FFP) with FFP infusions (Rock et al. 1991). PEX/FFP was superior to FFP infusion, with response rates of 47 % after seven procedures (one cycle) and of 78 % at 6 months, versus 25 % and 49 % in the infusion arm, respectively. Mortality at 6 months in the PEX/FFP arm was 22 % compared with 36 % in the plasma infusion arm. In a parallel study, Bell and coworkers (Bell et al. 1991) treated 108 patients either with PEX and corticosteroids ($n = 54$) or with corticosteroids alone, depending on the severity of disease. Overall survival was 91 %. Relapse was the predominant problem and occurred in 64 % of patients. Relapse was responsible for all deaths observed (10/108 patients), indicating that supplementation of plasma therapy with corticosteroids is associated with higher survival rates than PEX/FFP alone. Corticosteroids alone were effective in 52 % of milder TTP cases. Additional support for the beneficial effect of adjunctive treatment with immunosuppressant drugs stems from indirect evidence from the Oklahoma TTP-HUS registry. Following the introduction of corticosteroids by default in 2005/2006 a reduction in treatment-associated complications in TTP patients with ADAMTS13 of <10 % was directly correlated with a reduction in the number of PEX/FFP sessions required to achieve remission (Som et al. 2012). PEX/FFP is now usually supplemented with corticosteroids (oral prednisone at 1–2 mg/kg body weight or 125–250 mg of intravenous

methylprednisolone) and has become the mainstay of TTP treatment, with survival rates of nearly 90 % (Kremer Hovinga et al. 2010). In addition, patients should receive folic acid because of the increased requirements of the proliferating erythropoiesis and megakaryopoiesis.

The recommended volume exchanged per PEX session is one volume of the patient's estimated plasma volume, which corresponds to 40 mL/kg body weight per session, although a number of centers initiate treatment with 1.5 volumes (60 mL/kg body weight) for the first one to three sessions, followed by exchanges of one plasma volume daily thereafter. PEX/FFP is continued until remission is achieved, defined as a platelet count $\geq 150 \times 10^9$/L on two consecutive days, normalized LDH activity, and no new manifestations of organ dysfunction. Disappearance of the ADAMTS13 inhibitor and recovery of ADAMTS13 activity is not seen in all patients and seems not to be mandatory for achieving remission. Discontinuation of PEX is the only way to know whether a stable remission has been achieved. Given the initially high numbers of early exacerbations, some centers taper rather than stop PEX abruptly, although tapering of PEX does not seem to reduce relapse rates (Bandarenko and Brecher 1998). When PEX is stopped, tapering of corticosteroids is usually started and completed within 6–12 weeks of the last PEX session.

More intensified PEX/FFP regimens should be reserved for severe cases or patients initially refractory to plasma treatment. Escalation beyond 1.5 plasma volumes per session is not recommended as process efficacy progressively declines with increased recycling of infused plasma. Alternatives are PEX twice per day or plasma infusions between PEX sessions. Although it is recommended to avoid (gross) platelet transfusions, platelets may be given for invasive procedures.

The high mortality without appropriate treatment creates an urgency to initiate treatment before the ADAMTS13 activity is available, thereby performing PEX also on patients without severe acquired ADAMTS13 deficiency, considered by certain experts as not having "true TTP". It should be kept in mind, however, that ADAMTS13 activity was not available for patient stratification in the landmark study by Rock et al. (1991), although it is often claimed that the study consisted mainly of "true TTP" patients because of the exclusion of patients not able to support plasma infusions as a result of severe renal insufficiency. ADAMTS13 was later determined in a subset of 41/102 (40.2 %) patients, of which most, but not all, were found to have mild to medium levels of ADAMTS13 inhibitors (between 0.2 and 2 BU/mL) (Tsai et al. 2001). In addition, the Oklahoma TTP-HUS registry has demonstrated that the response to PEX in idiopathic TTP patients was similar in the subgroup without severe ADAMTS13 deficiency to those with severe ADAMTS13 deficiency (Kremer Hovinga et al. 2010; Vesely et al. 2003).

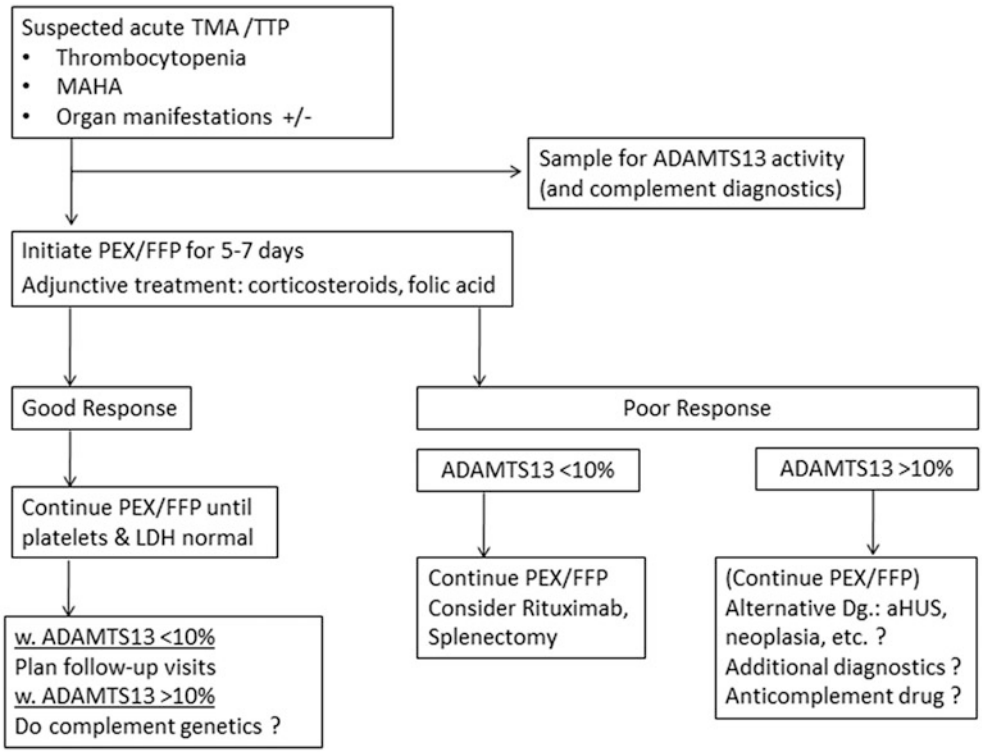

Fig. 2 TMA treatment algorithm. Adapted from (Cataland and Wu 2014)

Therefore, all patients clinically diagnosed with idiopathic TTP should be subjected to PEX/FFP treatment for 5–7 days. After this time, the ADAMTS13 activity result should be back from the laboratory and can help to guide next steps (see Fig. 2).

The initial infusion and PEX studies used fresh frozen plasma as replacement fluid (Rock et al. 1991; Bell et al. 1991). Subsequently, different plasma products (e.g., cryo-poor plasma, solvent/detergent plasma, and amtosalen UVA-treated plasma) were compared with FFP in small studies and found to be equivalent in treating TTP (Mintz et al. 2006; Pehta 1996; Scully et al. 2007).

Rituximab and Splenectomy

Rituximab is a humanized monoclonal antibody against CD20, a B cell surface marker expressed in increasing concentrations from late pro-B cells to mature B cells, but not present on plasma blasts or plasma cells. Rituximab was approved for the treatment of non-Hodgkin's lymphomas but is increasingly used for off-label indications, particularly to treat various autoimmune disorders. It rapidly depletes over 95 % of CD20-positive B cells from the circulation, thereby reducing autoantibody production.

First reports on the use of rituximab to treat acquired TTP appeared 2002 (Chemnitz et al. 2002; Gutterman et al. 2002). Although there are no randomized controlled trials, rituximab has since been used in several TTP cohorts of 30–40 patients as first-line treatment within the first days of presentation supplementing PEX/FFP (Scully et al. 2011; Westwood et al. 2013); in patients with insufficient response or refractory to PEX/FFP (Froissart et al. 2012); to treat relapses; and preemptively to correct ADAMTS13 values <10 % in remission to prevent future relapses (Hie et al. 2014; Westwood et al. 2013). All cases demonstrated a reduction in the relapse rate to 10–25 % and/or to postpone relapses, which again were successfully treated with rituximab (Scully et al. 2011; Froissart et al. 2012; Westwood et al. 2013; Hie et al. 2014). Scully et al. (2011) treated 40/86 (46.5 %; females 65 %) patients presenting with acute acquired TTP (34 de novo, 6 relapsed) with rituximab within 3 days of presentation, and observed shorter hospitalization times and a reduced relapse rate compared with 40 historical controls (females 82.5 %; relapsed 22.5 %). Unfortunately, the outcome of the remaining 46 patients, labeled as screen failures, was not reported. Because long-term follow-up data are missing, it is unknown, whether upfront rituximab prevents chronic relapsing TTP or (only) postpones relapses. Confirmation in a prospective randomized controlled trial is eagerly awaited.

Treatment escalation in patients refractory to or exacerbating on PEX/FFP is more readily accepted, as the treating physician is required to modify therapy. In the pre-rituximab era, these patients often received vincristine or cyclophosphamide.

The most difficult decision, given the lack of prospective randomized controlled trials and possible overtreatment, is whether patients in remission (with reappearance of anti-ADAMTS13 antibodies and severe ADAMTS13 deficiency) should be treated preemptively with rituximab to correct ADAMTS13 deficiency and hopefully prevent possible relapses (Westwood et al. 2013; Hie et al. 2014). Of note, a severe ADAMTS13 deficiency alone does not (always) seem to be sufficient to bring about acute TTP episodes. Jin et al. (2008) reported severe ADAMTS13 deficiency in 6/13 (46.1 %) patients on long-term follow-up without occurrence of relapses. A similar observation was reported for the Oklahoma TTP-HUS registry (Lim et al. 2015). Survivors of acute TTP episodes with severe ADAMTS13 deficiency are taking part in long-term follow-up programs with regular ADAMTS13 activity measurement. Of 52 patients in this program, 20 (38.5 %) had at least on one occasion in remission a severe ADAMTS13 deficiency, which recovered spontaneously in 75 % of cases. Six patients experienced a relapse on average 4 years after documentation of a severe ADAMTS13 deficiency.

Possible overtreatment is an important aspect because, although rare, the risk of progressive multifocal leukoencephalopathy (PML) seems to be higher in rituximab-treated patients with autoimmune disorders (5/100,000) than in the normal population (0.3/100,000) although no causal association has yet been proven (Molloy and Calabrese 2012). In addition, repeated administration of rituximab has been associated with the development of hypo-gammaglobulinemia in a considerable proportion of patients (Hie et al. 2014; Kaplan et al. 2014; Makatsori et al. 2014) and is associated with increased risk of serious infection (Kaplan et al. 2014; Makatsori et al. 2014). In this context, note that it has not been formally studied whether less-intensive rituximab treatment (i.e., only one or two weekly infusions instead of four) in TTP would result in similar responses as the current regimens employed. The presence of immune complexes between ADAMTS13 and anti-ADAMTS13 antibodies months to years into remission after an acute TTP episode (Ferrari et al. 2014) could represent the missing link to the observed long-term morbidity and reduced life-expectancy in survivors of acute TTP episodes with severe ADAMTS13 deficiency (Deford et al. 2013) and vindicate more aggressive treatment of the ongoing autoimmune response in the future.

In most studies, patients receive four weekly doses of rituximab (375 mg/m^2), following the typical B cell neoplasia regimen of rituximab every seven days. The French TMA reference network has adapted this scheme and administers four doses of rituximab within 15 days from the day a patient

is considered refractory to plasma treatment. In acute TTP, PEX/FFP has usually to be continued following the first rituximab administration(s). Rituximab is removed by PEX. McDonald et al. found that the median fall in rituximab level per PEX session was 65 %, although all patients achieved CD19-positive B cell counts of <1 % (McDonald et al. 2010). Therefore, rituximab is usually given immediately following a PEX session and the next session is protracted as much as possible and timed the next day.

Besides rituximab, splenectomy has been successfully employed in refractory and relapsing TTP for decades. Splenectomy reduces the relapse rate by 85 % and often leads to durable remission (Kremer Hovinga et al. 2004b; Kappers-Klunne et al. 2005; Dubois and Gray 2010), even in rituximab-pretreated patients. It has been shown that the spleen harbors a notable reservoir of pathogenic ADAMTS13-specific switched memory B cells and plasma blasts in frequently relapsing TTP patients treated with or without rituximab (Schaller et al. 2014), providing a rational for splenectomy in frequently relapsing TTP patients.

Personal Approach and Management of TMA and TTP Patients

TTP is a medical emergency with a high mortality rate if appropriate treatment is not quickly initiated. I initiate PEX/FFP in a patient with thrombocytopenia and MAHA (schistocytes on the blood smear, negative direct antiglobulin test, elevated levels of LDH, bilirubin, reticulocytes, and a haptoglobin level below the detection limit) without an apparent alternative explanation for the findings (clinical history, exclusion of disseminated intravascular coagulation).

PEX/FFP has to be performed on several consecutive days; therefore, placement of a central venous catheter is necessary in most cases to facilitate the high volume of plasma exchanged at high rate flow rates. Formally, PEX/FFP can be performed on plasma filtration systems as well as on centrifugal machines. The latter can pack red cells in the machine to a hematocrit of 80 % or higher (compared with 35–60 % in filtration systems) and remove >80 % of plasma. They process ~1.5 patient blood volumes to remove 1.2 plasma volumes, whereas filtration systems need to process three to four patient blood volumes to achieve similar plasma removal. Of note, all published treatment series in TTP have used centrifugal separators (Szczepiorkowski et al. 2010). Furthermore, red cells in TTP are often fragile, particularly at presentation of an acute episode, and tend to be more readily damaged on the membranes of filtration systems. Furthermore, in centrifugal separators, citrate can be used as anticoagulant (compared with heparin in filtration systems), which is a further advantage in severely thrombocytopenic patients. To prevent citrate accumulation and toxicity, which is particularly important when replacing large volumes using FFP, we add a calcium infusion to the return line.

Plasma is currently the only source of ADAMTS13. In TTP, PEX is universally performed with replacement of large volumes of plasma. Which donor plasma product is used has little importance as all plasma products are equally efficacious in TTP. The product used at our hospital for years was FFP, with the addition of solvent/detergent plasma since about 2015. Given the high incidence of an underlying autoimmune pathophysiology in TTP, patients receive corticosteroids (typically 1–2 mg/kg body weight) in addition to PEX/FFP and, as supportive measures, folic acid and a proton-pump inhibitor.

PEX/FFP is performed until a normal platelet count ($>150 \times 10^9$/L) is documented on two consecutive days. Usually, the patient is supervised for a further 2–3 days in hospital. Tapering of steroids is started and is complete after 6–12 weeks. During the first few months, outpatient follow-up with laboratory investigations is every week, then once a month for 2–3 months, and then every 3 months. After 1 year, follow-up visits were extended to once every 6 months and after 2 years to once a year. Recent findings of reduced life expectancy and high morbidity in TTP survivors, as well as more widespread use of preemptive rituximab treatment in patients with reappearance of severe acquired ADAMTS13 deficiency, has resulted in a change in our follow-up program. We now recommend follow-up visits every 3 months long-term, beyond the first year.

Before the administration of any blood products, a sample should be withdrawn for ADAMTS13 activity (and possibly complement factor) determination; however, PEX/FFP should not be delayed until the results are available. My decision to initiate PEX is thus not based on ADAMTS13 activity, but on clinical criteria (platelet count, MAHA, neurologic symptoms) although ADAMTS13 activity level is used as supportive evidence, especially during management and to plan patient follow-up, as relapses happen primarily in patients who initially presented with a severe ADAMTS13 deficiency (Kremer Hovinga et al. 2010). During follow-up, ADAMTS13 activity determination is standard at our center and patients often demonstrate fluctuating levels of ADAMTS13 activity; we have seen spontaneous recovery of severe ADAMTS13 deficiency in two patients. Consequently, so far we have not preemptively treated patients with severe ADAMTS13 deficiency without overt TTP.

Fairly typical in severe acquired ADAMTS13 deficient TTP is the initial response to PEX/FFP, with a clear increase or even normalization of the platelet count, which then drops again, sometimes together with new signs of tissue/organ ischemia after about 1 week. Re-investigation of ADAMTS13

inhibitors typically demonstrates an increase in the inhibitor titers (Fontana et al. 2004), a phenomenon sometimes referred to as inhibitor boosting. This seems to be unique to acquired TTP and is not seen in other autoimmune disorders treated with PEX. Many of these patients become plasma-refractory and require escalation of treatment. A more intense PEX/FFP regimen with increased replacement volume per session (from 1 to 1.5 plasma volumes) and plasma infusions between PEX/FFP sessions is an option, but nowadays these patients usually receive rituximab. As in most other centers, I use the dose established for B cell neoplasias (i.e., 375 mg/m^2) and usually give a complete course of four weekly doses. Because PEX/FFP needs to be continued, at least initially, and rituximab is removed by PEX/FFP, rituximab is administered directly after a PEX/FFP session, and the subsequent PEX/FFP session postponed until the next day to allow as large as possible an interval. Despite the fact that a considerable amount of rituximab is removed by the next PEX/FFP session, complete depletion of peripheral CD19-positive B cells is achieved in virtually all patients.

In patients with an acute TMA episode and a normal or only mildly reduced ADAMTS13 activity and (pronounced) renal involvement, anti-complement treatment is given, especially in children and young adults. In the absence of renal involvement, PEX/FFP is continued for at least another week and the search for an underlying disorder, mainly occult neoplasia, is intensified.

As pointed out before, survivors of acute episodes of TTP and severe acquired ADAMTS13 deficiency are at high risk of subsequent relapse, which occurs in nearly half the patients (Kremer Hovinga et al. 2010). Once a patient presents with a relapse, splenectomy or a more intense immunosuppressive regimen with rituximab, is used to prevent further relapse. Our in-house results on long-term relapse prevention are better with splenectomy, but I leave the choice to the patient, and most prefer to try rituximab first. Rituximab is chosen when other autoimmune disorders (i.e., systemic lupus erythematosus) are present in addition to acquired TTP. During follow-up visits after the first acute episode these possibilities are discussed with the patient, who is vaccinated against pneumococcal and meningococcal infection, as well as *Haemophilus influenza,* as soon as corticosteroids have been sufficiently reduced.

Treatment of Hereditary TTP and aHUS

In hereditary TTP, acute episodes can be effectively prevented by regular plasma infusions every 2–3 weeks (Upshaw 1978). However, the clinical course in the majority of hereditary patients is less predictable except for patients with the p.R1060W mutation, who seem to be largely asymptomatic outside special situations of increased risk such as pregnancy, heavy alcohol intake, or (severe) infection. Although some patients remain asymptomatic for years, others have frequent bouts. The initial notion that, once a first acute episode has occurred, frequent relapses follow does not seem to be true for all patients, making it difficult for the treating physician to decide when to start plasma-prophylaxis to prevent long-term morbidity.

In contrast to hemophilia, where one-third of patients regularly treated with FVIII-containing products develop inhibitory alloantibodies, so far only 1 of about 120 congenital ADAMTS13-deficient patients on regular plasma prophylaxis has been reported to have developed a treatment-relevant inhibitory alloantibody (Raval et al. 2014). Noninhibitory anti-ADAMTS13 antibodies have been observed in some patients on regular prophylaxis; however, these antibodies do not seem to interfere with ADAMTS13 recovery or ADAMTS13 plasma half-life (Fujimura et al. 2011; Kremer Hovinga and Meyer 2008).

Early administration of plasma or plasma-containing blood products is important for successful pregnancy outcomes in hereditary TTP: Fujimura and colleagues reported 15 pregnancies in nine USS patients, resulting in seven (47 %) live births, all preterm except for one in a woman on plasma prophylaxis (Fujimura et al. 2011). Scully and colleagues reported on eight miscarriages and 54 pregnancies in 23 USS patients, six of whom were homozygous and 17 compound heterozygous carriers of ADAMTS13 p.R1060W. Successful outcomes with live-births were noted in 36 (58 %) pregnancies. In 26/36 successful pregnancies, patients had received plasma treatment (Scully et al. 2014). In a population with a somewhat different ADAMTS13 mutational spectrum, von Krogh and coworkers reported a successful outcome of 6/15 (40 %) pregnancies in nine patients (von Krogh et al. 2014).

For many years, plasma therapy was the only option in aHUS because of the lack of anti-complement therapy. Today, the first anti-complement agent, eculizumab, is available and inhibits activation of C5 to C5a and C5b, thereby preventing generation of the terminal complement complex C5b-9. Ecuizumab has been approved by the FDA and EMA for the treatment of aHUS, diagnosed on nonspecific criteria (acute TMA with ADAMTS13 >5 %). Following a number of case reports, prospective studies in predominately adult aHUS patients with long diseases courses, chronic kidney disease, and often overt TMA dependent on regular PEX showed eculizumab to be well tolerated and (very) effective; many patients were able to halt regular plasma therapy (Legendre et al. 2013; Licht et al. 2015).

More single case reports and small case series have been published on successful anti-complement treatment in the absence of complement mutations, in Stx-associated HUS,

transplant rejection, allogenic stem-cell transplantation-associated TMA, and even hereditary TTP. The use of eculizumab outside clinical trials should currently be restricted to patients with an established diagnosis of aHUS, preferably with identified underlying complement mutation(s). The high treatment costs, the fact that eculizumab has to be continued for a very long time (possibly indefinitely), as well as the increased risk of meningococcal infection (requiring vaccination before treatment initiation) all need to be considered.

Novel Drugs Under Evaluation

Von Willebrand Factor A1 Domain Targeted Therapy

Platelet–von Willebrand factor (VWF) interaction is a central paradigm in TTP. Involved moieties are glycoprotein Ib/IX on platelets and the VWF A1 domain. To date, two compounds blocking the VWF A1 domain have been tested in acquired TTP. Development of the first compound, ARC1779, an aptamer specifically binding to the VWF A1 domain, was halted after early termination of the phase II trial as a result of difficulties in patient recruitment (intention to enroll 100, terminated after nine patients were enrolled) and financial issues. Although only seven TTP patients had received ARC1779, proof of principle for the VWF A1 domain blocking approach and valuable information were gained (Jilma-Stohlawetz et al. 2011; Cataland et al. 2012).

The second compound, capalcizumab, is derived from single-chain antibodies naturally occurring in Camelidae. It was assessed in a phase II, multicenter, randomized placebo-controlled study in patients with acquired TTP (the TITAN trial). After randomization (1:1) patients received, besides standard of care, either capalcizumab or placebo in conjunction with, and for 30 days following daily PEX/FFP. Time to platelet recovery was significantly shorter in the capalcizumab compared with the placebo arm and biomarkers reflecting ischemic organ damage tended to normalize more rapidly (Peyvandi et al. 2016). The incidence of exacerbations (defined as recurrence of thrombocytopenia during the period of daily PEX/FFP or within 30 days of stopping PEX/FFP) was reduced in the capalcizumab arm, despite the fact that many patients had persistent severe ADAMTS13 deficiency. The results indicate that the anti-VWF A1 domain compound protects against platelet consumption in the microvasculature. Because the underlying autoimmune disorder is not treated, the increased relapse rate after stopping caplacizumab in this arm was not a surprise. Given the mode of action, bleeding-related adverse events were more common in the caplacizumab arm (53 % versus 38 % in the placebo arm); however, most bleeding events were mild and did not require intervention. Serious bleeding events were similar in both treatment arms. Caplacizumab is currently being further evaluated in a phase III study, the HERCULES trial (clinicaltrials.gov NCT02553317).

Recombinant ADAMTS13 (BAX930)

Lack of ADAMTS13 is the hallmark of hereditary TTP and of most cases of acquired TTP. Early in vitro experiments on hereditary TTP (Antoine et al. 2003) and acquired TTP (Plaimauer et al. 2011) demonstrated that addition of recombinant ADAMTS13 was an effective measure for restoring VWF-cleaving activity in these plasmas, even in the presence of ADAMTS13 inhibitors, where the required amount of recombinant ADAMTS13 was dependent on the titer of the functional ADAMTS13 inhibitor. Subsequently, effectivity and feasibility were proven in various animal models of hereditary and acquired TTP (Schiviz et al. 2012; Plaimauer et al. 2015; Tersteeg et al. 2015). A phase I trial of recombinant ADAMTS13 (BAX930) in patients suffering from hereditary TTP has been performed in 2016 and results are awaited (clinicaltrials.gov NCT02216084).

Anti-complement Agents

A number of studies are underway with different anti-complement agents in aHUS and other complement-mediated disorders. These agents can act through blocking C5 cleavage by C5 convertase (e.g., coversin, which is also effective in patients resistant to eculizumab as a result of *C5* polymorphisms; clinicaltrials.gov NCT02591862), blocking interaction of C5a with its receptor C5aR (e.g., CCX168; clinicaltrials.gov NCT02222155 and NCT02464891), interfering with the lectin pathway (e.g., OMS721, a fully human monoclonal antibody targeting MASP-2; clinicaltrials.gov NCT02222545), or selectively inhibiting components of the classical complement pathway (e.g., TNT009, a selective inhibitor of C1s; clinicaltrials.gov NCT02502903).

Outlook

Evidently, the pathophysiological insights into TTP and HUS gained during the past two decades have already had a tremendous impact on treatment and outcome in affected patients and have helped to remove some of the grimness of these rare diseases. There is, however, ample room for improvement as mortality and long-term morbidity are still considerable. The prospect of a number of promising novel therapies under investigation is very stimulating.

Severe ADAMTS13 deficiency in remission in patients with acquired TTP is a strong risk factor; however, spontaneous recovery of ADAMTS13 activity has been observed in a number of patients (Jin et al. 2008; Lim et al. 2015), and often

a second hit such as infection, regnacy or other events reflected by formation of neurtophile extracellular traps is needed to set off an acute TTP episode (Fuchs et al. 2012; Miyata and Fan 2012). To prevent overtreatment, biomarkers to identify patients at risk of imminent relapse would be highly desirable. This is also true for the positive confirmation of aHUS diagnosis with appropriate biomarkers and identification of patients who would benefit from long-term anti-complement therapy or for whom tapering of such agents would be safely possible. The ultimate goal is to further advance therapeutic regimens in TTP and HUS, thereby improving daily life, long-term outcome, and morbidity in affected patients.

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Take Home Messages

1. Acute thrombotic microangiopathies are life-threatening disorders characterized by (severe) thrombocytopenia and microangiopathic hemolytic anemia. Primary forms, thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS), must be distinguished from secondary forms with underlying conditions or disorders.
2. ADAMTS13 activity <10 % of that in normal plasma:
   - Proves a diagnosis of TTP
   - Identifies patients at high risk of relapse
   - ADAMTS13 functional inhibitor or ADAMTS13 antibodies help to distinguish between acquired and hereditary TTP
   - Presence of an ADAMTS13 functional inhibitor or ADAMTS13 antibodies provides the rational for immunosuppressive treatment (corticosteroids, rituximab, splenectomy)
3. At present, ADAMTS13 activity >10 % of normal does not rule out the need for plasma exchange; however, alternative diagnoses have to be looked for and anti-complement treatment should be considered in cases with prominent renal injury.
4. HUS may follow infection by enterohemorrhagic bacteria (diarrhea-positive HUS) or result from excessive activation of the alternative complement pathway as a result of mutations in complement factors or complement regulators.

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Thrombocytosis and Essential Thrombocythaemia

Search Strategy and Evidence-Based Diagnosis

Samah Alimam and Claire N. Harrison

Abstract

Thrombocytosis is a common occurrence in general medical and surgical patients and can have a primary or secondary cause. Investigating a patient with thrombocytosis requires a systematic approach. Secondary thrombocytosis is often self-limiting and not associated with any thromboembolic complication or vasomotor symptoms. Essential thrombocythaemia is a clonal myeloproliferative neoplasm, which is associated with broad clinical sequelae and serious complications such as haemorrhage and thrombosis. Discovery of the *JAK2*-V617F, *MPL* and *CALR* genes has deepened understanding of the pathogenesis of the disorder as well as providing targets for therapy and guidance for prognosis. Subjects with essential thrombocythaemia are risk stratified and managed accordingly. Unless contraindicated, antiplatelet therapy is given to all patients and cytoreductive therapy is reserved for those who are high risk (i.e., over the age of 60 years or with previous history of a thromboembolic event).

Introduction

Platelets, first noted by Bizzozero in 1882, are discoid anucleate cell fragments circulating in the peripheral blood. They are responsible for maintaining vascular integrity, haemostasis and assisting with the acute and chronic inflammatory response (Ribatti and Crivellato 2007). Through commitment of multipotent haemopoietic stem cells to the megakaryocyte lineage, circulating platelets are produced, a relationship first described by James Wright in 1906 (Deutsch and Tomer 2006). Haemopoietic stem cells give rise to the early myeloid progenitors; megakaryocytes develop from a committed proliferating progenitor, a colony-forming unit-megakaryocyte. Once the colony-forming unit-megakaryocytes stop proliferating, they enter endomitosis (undergo DNA replication in the absence of cellular division), giving rise to classical polyploid cells with a single polylobulated nucleus – mature megakaryocytes (Cazzola 2008).

With increasing access to diagnostic techniques in medicine, thrombocytosis is a commonly encountered clinical phenomena; the defined threshold for clinically significant thrombocytosis is accepted as a sustained platelet count greater than 450×10^9/L (Schafer 2004; Harrison et al. 2010). Depending on pathogenesis, it can be defined as primary, secondary or, in a small number of individuals, as hereditary thrombocytosis (Fig. 1). Because the diagnosis has implications for long-term management and prognosis, it is imperative to establish the cause in a subject with a persistent thrombocytosis.

Platelet production is regulated by the action of thrombopoietin interacting with its receptor. Thrombopoietin has a central role in megakaryocytopoiesis (platelet production) and thrombocytosis. Through binding to the thrombopoietin receptor, MPL (also termed c-MPL; cellular homologue of retrovirus complex inducing myeloproliferative leukaemia, CD110), thrombopoietin signals to the haemopoietic stem cells to commit to the megakaryocyte progenitor cell line and stimulates the proliferation and differentiation of megakaryoblasts into megakaryocytes. The thrombopoietin

The data in this chapter is supported by a Medline search using the following key words: platelets, thrombocytosis, thrombopoietin, essential thrombocythaemia, myeloproliferative neoplasms, *JAK2*, *CALR*, *MPL*, epigenetics, risk stratification, thrombosis, haemorrhage and cytoreductive therapy.

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Fig. 1 Causes of thrombocytosis (Harrison et al. 2010). Diagram illustrating differential diagnosis of thrombocytosis: *green* clonal causes for thrombocytosis, *red* reactive causes for thrombocytosis, *yellow* spurious causes of thrombocytosis, *blue* hereditary thrombocytosis. *RARS* refractory anaemia with ring sideroblasts, *MDS* myelodysplastic syndrome, *MPN-U* myeloproliferative neoplasms – unclassified

receptor is expressed on CD34-positive cells, megakaryocytes, platelets and endothelial cells. Platelet production is driven by the combined effects of satisfactory thrombopoietin gene expression, thrombopoietin concentration and adequate number of c-MPL-bearing cells (Fig. 2a). Thrombopoietin is expressed primarily by the liver, but also by the kidney, spleen and bone marrow. When platelet numbers are low, plasma levels of thrombopoietin increase to expand megakaryocytopoiesis and platelet production (Fig. 2b, c); thrombopoietin enhances megakaryocyte proliferation through JAK2 (Janus kinase 2) and STAT5 (signal transducer and activator of transcription 5) activation. Likewise, when the platelet count rises, more thrombopoietin binds to platelet c-MPL receptors and less ligand is available for megakaryocyte receptors, leading to slowed megakaryocytopoiesis (i.e., negative feedback loop) (Cazzola 2008).

In the normal state, liver is the predominant thrombopoietin-producing organ, with little influence from external signals (Jelkmann 2001). In steady state, the plasma level of thrombopoietin is low at 10–12 mol/L. Unlike hepatic thrombopoietin, bone marrow stromal cells respond to thrombocytopenia and upregulate thrombopoietin gene expression. Functional thrombopoietin receptors remove thrombopoietin via absorption and internalisation of the cell surface complex.

Secondary Thrombocytosis

The key driver of secondary thrombocytosis is often an inflammatory or reactive process. Patients are usually diagnosed coincidentally and are often asymptomatic. Secondary thrombocytosis has a lower incidence of complications than primary thrombocytosis. In a study of 890 subjects, Rose and colleagues described secondary thrombocytosis to be the principal diagnosis in over 94 % of subjects, with an infectious aetiology contributing to over 50 % of cases (Rose et al. 2012). Because platelets are key acute-phase reactants, their number increases in response to various stimuli, including infections, inflammatory conditions and bleeding, all of which can cause a short-term elevation of platelet count. In chronic infections, iron deficiency and neoplasms, the thrombocytosis may be more persistent (Griesshammer et al. 1999). Secondary thrombocytosis in children is also driven by similar reactive causes, although in certain population groups haemolysis secondary to thalassemia or sickle cell disease must be also be considered. As the differential diagnosis is broad, it is not surprising that any investigation of thrombocytosis must begin with exclusion of potential secondary causes, as outlined in Fig. 1; this requires a though history and examination of the patient, in addition to review of all full blood count and biochemistry parameters before exclusion of an underlying clonal disorder.

The pathophysiology of secondary thrombocytosis is as varied as the differential diagnosis. The inverse relationship between platelet count and thrombopoietin in healthy subjects is lost in secondary thrombocytosis, where levels of the hormone are higher than expected (Cerutti et al. 1997). A number of studies have suggested that thrombopoietin behaves as an acute-phase protein and its increase is responsible for thrombocytosis (Ceresa et al. 2007). Furthermore, acute-phase inflammatory mediators such interleukin (IL)-1, IL-4, IL-6, IL-11 and tumour necrosis factor (TNF) are all involved in stimulating megakaryocytopoiesis via hepatic

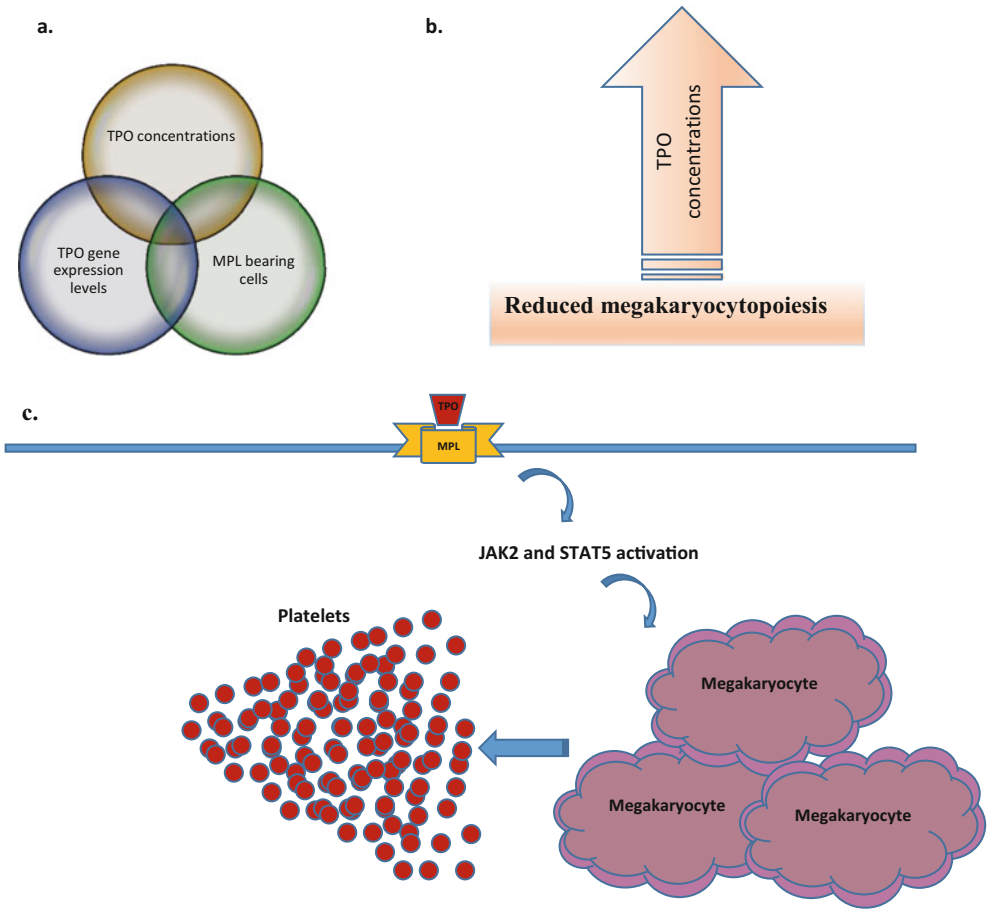

Fig. 2 (**a**) Key components in platelet production and thrombocytosis. (**b**) Response of thrombopoietin to reduced megakaryocytopoiesis. (**c**) Action of thrombopoietin on MPL, leading to megakaryocyte proliferation and platelet production. *TPO* thrombopoietin, *MPL* thrombopoietin receptor, *JAJ2* Janus kinase 2, *STAT5* signal transducers and activators of transcription 5

thrombopoietin production (Avraham 1993; Ceresa et al. 2007). A number of studies have demonstrated that subjects with secondary thrombocytosis have elevated levels of IL-6, C-reactive protein (CRP), ferritin and other acute-phase reactants compared with clonal thrombocytosis. Tefferi and colleagues noted that over 75 % of subjects with secondary thrombocytosis had elevated CRP levels compared with 10 % of clonal thrombocytosis patients (Tefferi et al. 1994). Therefore, thrombopoietin behaves as an acute-phase protein and is involved in thrombocytosis of inflammatory disorders; other acute-phase reactants cooperate with thrombopoietin in inducing an elevated platelet count (Ceresa et al. 2007) (Fig. 3).

Iron deficiency is a common cause of thrombocytosis; although the pathophysiology remains unclear, elevated erythropoietin levels have been reported (Akan et al. 2000; Balcik et al. 2002). A number of investigators have assessed the effect of erythropoietin on the thrombopoietin receptor (MPL). Although it does not interact directly with MPL, erythropoietin may enhance the effect of thrombopoietin, stimulating platelet production (Broudy et al. 1995; Balcik et al. 2002). Routine investigations to exclude an underlying iron deficiency are therefore fundamental in any diagnostic work-up for thrombocytosis; however, the presence of iron deficiency does not exclude clonal thrombocytosis.

Although, the clinical complications seen with primary thrombocytosis are less frequent in reactive thrombocytosis, a number of studies have shown that thrombocytosis is an independent risk factor for thromboembolic events in cancer subjects (Zakai et al. 2004; Khorana et al. 2005). Not surprisingly, management of secondary thrombocytosis centres on treating the underlying cause and management of any potential complications.

Spurious Thrombocytosis

Spurious thrombocytosis is a rare cause of elevated platelet count; it is characterised by the presence of platelet-like structures in the peripheral blood. Some automated blood counters report these structures as platelets and therefore produce an incorrect count. Many pathogens, cryogloblins and red blood cell fragments can be misread as platelets. A peripheral blood smear and thorough history should be sufficient to make this diagnosis.

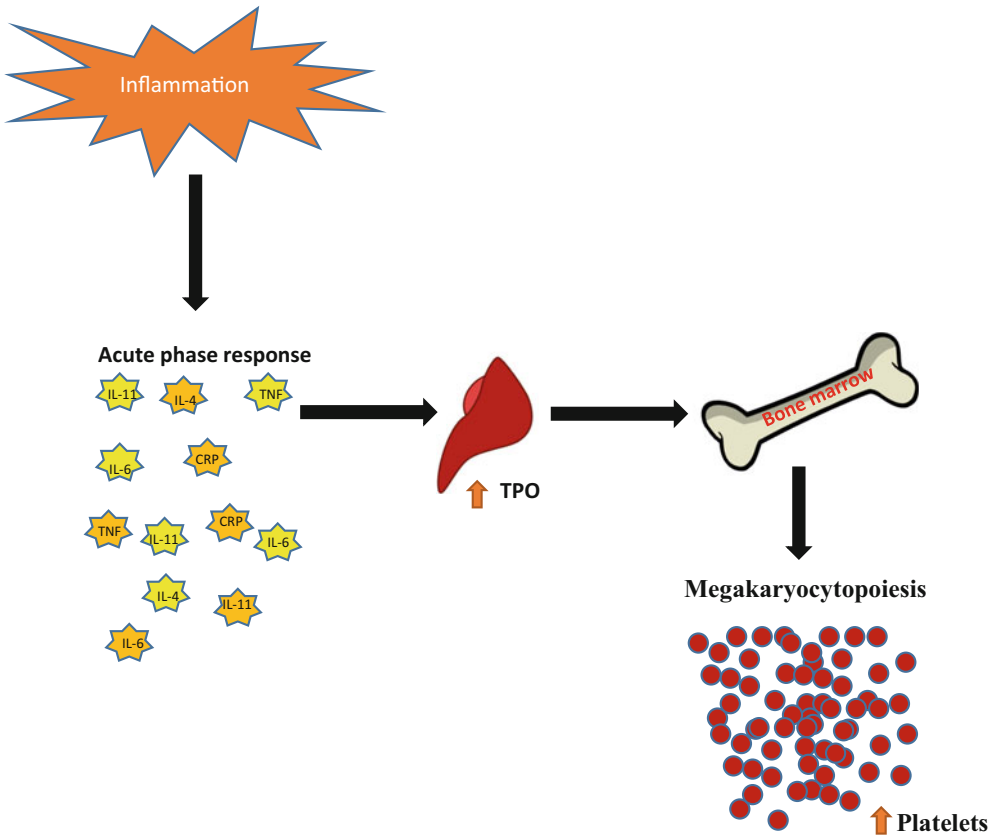

Fig. 3 Proposed mechanism for reactive thrombocytosis. Proposed mechanism of increased thrombopoietin (*TPO*) production in response to inflammatory mediators such as IL-2, IL-4, IL-6, IL-11, tumour necrosis factor (*TNF*) and C-reactive protein (*CRP*), resulting in enhanced megakaryocytopoiesis and increased platelet production

Hereditary Thrombocytosis

Hereditary thrombocytosis (HT) is a polyclonal, heterogeneous autosomal dominant disorder that results in overactivation of the thrombopoiesis pathway, resulting in thrombotic and haemorrhagic complications similar to those seen in essential thrombocythaemia (Skoda and Prchal 2005). Germline mutations in families with hereditary thrombocythaemia have been identified in the *MPL* gene encoding the thrombopoietin receptor. The first *MPL* mutation described was that of substitution of serine in position 505 with asparagine (S505N) in the transmembrane domain of MPL protein, discovered in a family with autosomal dominant thrombocytosis (Skoda 2009). The other commonly described mutations are outlined in Table 1.

The mutations in *MPL* result in loss of the inhibitor upstream reading frames, resulting in increased production of thrombopoietin and, consequently, of platelets (Ghilardi and Skoda 1999). Not all mutations arise within the thrombopoietin receptor or *MPL* gene. Mead and colleagues reported a germline *JAK2*-V617L mutation in a British patient with thrombocytosis and cardiovascular events, and no bone marrow changes suggestive of myeloproliferative neoplasms (Mead et al. 2012); this highlights the number of potential gene mutations contributing to thrombocytosis. However, less than 10 % of pedigrees have a known mutation because, although some disease-causing genes have been reported, the majority remain unidentified (Skoda 2009). There have been infrequent reports of progression

Table 1 Common mutations described in hereditary thrombocytosis (Skoda 2009; Mead et al. 2012)

Gene	Mutation
MPL	S505N
MPL	K39N
MPL	P106L
TPO	Substitution, splice donor site, intron 3
TPO	Deletion in 5′ UTR
TPO	Substitution, exon 3
TPO	Substitution, intron 3
JAK2	V617L

MPL thrombopoietin receptor, *TPO* thrombopoietin, *JAK2* Janus kinase 2, *UTR* untranslated region

of thrombocytosis to myelofibrosis and acute myeloid leukaemia in subjects with HT (Posthuma et al. 2010)

Clonal Thrombocytosis

Figure 1 outlines the differential diagnosis of clonal thrombocytosis. Clonal thrombocytosis should be a diagnosis of exclusion when there is persistent thrombocytosis in the absence of reactive causes and/or presence of a clonal marker. The following section focusses on myeloproliferative neoplasms and essential thrombocythaemia.

Myeloproliferative Neoplasms

Myeloproliferative neoplasms (MPNs) are characterised by clonal expansion of the different myeloid cells, the commonest forms being essential thrombocythaemia (ET), polycythaemia vera (PV), primary myelofibrosis (PMF) and Philadelphia-positive chronic myeloid leukaemia (CML), in addition to the other disorders outlined in Fig. 4. Although all MPN disorders are phenotypically diverse, thrombocytosis can be a feature of any MPN. During the last 20 years there have been major advances in the understanding of MPNs and description of a number of acquired mutations that drive disease pathogenesis. This chapter focusses on ET; however, it is important in the diagnostic work-up to consider all the MPNs.

The MPNs overlap in phenotype and mutational drivers. CML is a MPN defined by the *BCR-ABL* fusion gene, arising from the Philadelphia translocation, and must not be missed in the diagnostic work-up of any thrombocytosis. PV and PMF share the same clonal markers as ET and are distinguished clinically, biochemically and histologically. The hallmark of PV is erythrocytosis with an elevated haematocrit, whereas PMF is characterised histologically by bone marrow fibrosis and, in advanced disease, profound constitutional symptoms.

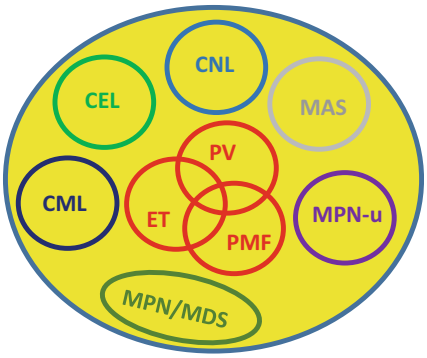

Fig. 4 Myeloproliferative neoplasms (Vardiman et al. 2009). *PV* polycythaemia vera, *ET* essential thrombocythaemia, *PMF* primary myelofibrosis, *CML* chronic myeloid leukaemia, *CNL* chronic neutrophilic leukaemia, *MAS* mastocytosis, *CEL* chronic eosinophilic leukaemia not otherwise specified, *MPN-U* myeloproliferative neoplasms – unclassified, *MPN/MDS* myeloproliferative neoplasm/myelodysplastic syndrome overlap

Essential Thrombocythaemia

Essential thrombocytosis (ET) was first described in 1934 by Epstein and Goedel. ET is a Philadelphia-negative MPN characterized by persistent thrombocytosis (Epstein and Goedel 1934). It is commonly diagnosed in the sixth decade of life and has an incidence of 0.4–2.5 per 100,000 per year (Rozman et al. 1991). ET has a higher prevalence in females, with a second peak in women in the third decade of life. ET is extremely infrequent in childhood; diagnoses in rare cases occur in the second decade of life (Dame and Sutor 2005). Life expectancy is normal and mirrors the general population in the first decade of diagnosis. However, ET is associated with thromboembolic complications, progression to myelofibrosis and acute myeloid leukaemia, with reduced life expectancy after the first decade (Wolanskyj et al. 2006; Barbui et al. 2011).

ET is characterised by clonal proliferation by mutually exclusive mutations in *JAK2*, calreticulin (*CALR*) or *MPL*; however, a proportion of subjects do not harbour an identified mutation and are termed "triple negative" (TN). The diagnosis of ET is based on the WHO criteria, which encompasses laboratory, molecular, clinical and histological criteria as outlined in Fig. 5. In addition to thrombocytosis, ET can be associated with an elevated white cell count, which has prognostic significance. ET must be distinguished from PV, early pre-fibrotic myelofibrosis and myelodysplastic syndrome. Low ferritin in the presence of normal haemoglobin levels points to a diagnosis of PV, whereas anaemia, splenomegaly and elevated lactate dehydrogenase, or a leucoerythroblastic peripheral smear with or without teardrop poikilocytes, suggest myelofibrosis. Bone marrow histology aids in differentiating between the three pathologies,

ET has a heterogeneous clinical phenotype, ranging from the asymptomatic patient to profound symptomology impacting considerably on quality of life. The clinical course of ET manifests with microcirculatory disturbances and arterial and venous thromboses (Landolfi et al. 2008). Microcirculatory disturbances, arising from platelet thrombi in end arterial circulations, result in vasomotor symptoms, often presenting as erythromelagia, headaches, visual disturbances and chest pain (van Genderen and Michiels 1997; Michiels et al. 2006). Splenomegaly, weight loss, fatigue and pruritus are other frequent clinical presentations.

I.	Sustained platelet count > 450 x 10^9/l.
II.	Bone marrow biopsy demonstrating proliferation of megakaryocytes with increased number of enlarged and mature forms. No significant increase of left shift of granulopoiesis or erythropoiesis.
III.	Not meeting WHO criteria for PV, PMF, CML, MDS or other myeloid neoplasm.
IV.	Presence of *JAK2*V617F, *CALR* or other clonal marker or no evidence of reactive thrombocytosis.

Fig. 5 WHO criteria for diagnosis of essential thrombocythaemia; all four components must be met (Vardiman et al. 2009). *PV* polycythaemia vera, *PMF* primary myelofibrosis, *CML* chronic myeloid leukaemia, *MDS* myelodysplastic syndrome

Arterial and venous thromboses are serious complications of ET and are described in up to 25 % of patients. Arterial thrombosis presenting as stroke or transient ischemic attack is the most common, although peripheral vascular and coronary artery thromboses are also well described. Venous thrombosis usually occurs in unusual sites such as cerebral sinus and splanchnic veins. The risk of thrombosis remains high throughout the disease state and risk of recurrent thromboembolic events is as high as 20 % (Wolanskyj et al. 2006). It is interesting to note that there is an absence of correlation between platelet count and vasomotor symptoms or thromboembolic complications (Campbell et al. 2012). Enhanced platelet, leukocyte and endothelial activation has been consistently demonstrated in ET subjects and is the proposed pathogenetic link between activated platelets and thrombotic complications (Patrono et al. 2013).

Thrombocytosis, paradoxically, is also associated with bleeding complications. Over 10 % of subjects develop a major haemorrhagic event, including gastrointestinal, intracranial and urogenital bleeding (Finazzi et al. 2012). A number of studies have demonstrated impaired platelet function in ET subjects (Finazzi et al. 1996; Trelinski et al. 2008), although these do not correlate well with bleeding risk. Furthermore, extreme thrombocytosis is a cause of acquired von Willebrand disease as a result of increased adsorption of the large multimers, which can further exacerbate the risk of bleeding.

Evolution of ET to post-ET myelofibrosis occurs in up to 30 % of subjects, with risk increasing after the second decade (Wolanskyj et al. 2006; Passamonti et al. 2008). Bone marrow histology is crucial in confirming the transformation. Progression to acute myeloid leukaemia, diagnosed in the presence of >20 % blast cells in the peripheral blood smear or bone marrow aspirate, is noted in less than 10 % of subjects; however, the risk increases with advancing age and with previous cytoreductive therapy (Kiladjian et al. 2006; Wolanskyj et al. 2006; Passamonti et al. 2008).

Histology

The peripheral film smear is usually normal in ET; any evidence of left shift, teardrops or dysplasia suggests an alternative diagnosis. Bone marrow histological confirmation of the underlying pathology remains integral in the diagnosis of MPNs and specifically in distinguishing between the three Philadelphia-negative MPNs in view of their overlapping phenotype. ET histology is characterised by the presence of single or clustered large to giant mature megakaryocytes with deeply lobulated staghorn-like nuclei, normal or slightly increased cellularity and the absence of or minimum reticulum fibrosis (Fig. 6) (Vardiman et al. 2009; Harrison et al. 2010). Dysplastic megakaryocytes, erythroid hyperplasia and significant reticulum fibrosis are against the diagnosis of ET and suggest a diagnosis of PV, PMF or myelodysplastic syndrome. Because of the subjective nature of bone marrow pathology and interpretation, it is advisable to avoid categorising into pre-fibrotic MF versus true ET unless other features are present. It is better to focus on establishing a firm diagnosis of one or other because it has a considerable impact on prognosis and management (Wilkins et al. 2008).

Janus Kinase 2

The Janus kinase family of tyrosine kinases includes *JAK1*, *JAK2*, *JAK3* and *TYK2*. JAK is characterized by four functional domains: FERM, SH2, pseudokinase (JH2) and the tyrosine kinase domain (JH1) (Fig. 7). The JH2 domain lacks kinase activity and therefore acts as a negative regulator of JAK protein kinase function (Jatiani et al. 2010). The JAK proteins play important roles in hematopoietic cell signalling by the cytokines, erythropoietin, thrombopoietin, granulocyte macrophage colony-stimulating factor, and granulocyte colony-stimulating factor receptors in hematopoietic cells. Signalling is activated when these receptors bind their

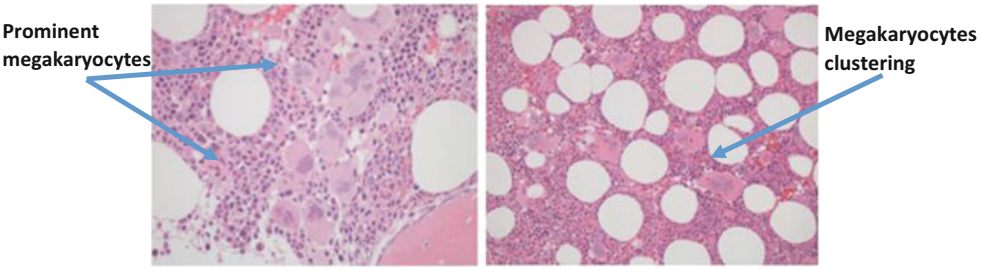

Fig. 6 Classical histological features of bone marrow trephine in essential thrombocythaemia, demonstrating prominent (*left*) and clustered (*right*) large megakaryocytes

Fig. 7 Janus kinase, demonstrating the four domains and site of *JAK2*-V617F mutation (Jatiani et al. 2010). *PK* phosphokinase, *TK* tyrosine kinase

cognate ligands (Spivak 2010), resulting in phosphorylation and subsequent activation of their downstream transcription factors, STATs. The activated STATs dimerise and translocate to the nucleus, where they activate or repress target gene promoters, as shown in Fig. 8 (Jatiani et al. 2010).

The acquired gain-of-function mutation in exon 14 of the *JAK2* gene, with a substitution of a valine for a phenylalanine at position 617 (V617F) in the negative autoregulatory JH2 pseudokinase domain (Baxter et al. 2005), has become instrumental in the understanding of MPN pathophysiology. The mutation disrupts the inhibitory role of the JH2 domain on the JH1 domain, whereby the activation loop of JH1 adopts a conformation such that it can be phosphorylated by an adjacent *JAK2*-V617F molecule (Baxter et al. 2005; Jatiani et al. 2010). This promotes cell proliferation and resistance to cell death, as well as hypersensitivity to hematopoietic growth factors and cytokines signalling through receptors utilising JAK (Spivak 2010).

The *JAK2*-V617F mutation is prevalent in up to 50 % of ET subjects (Antonioli et al. 2005). More recently, mutations outside of exon 14 and 12 (more commonly seen in PV), *JAK2*-V625F and *JAK2*-F556V, have been discovered in subjects with ET. Although both are gain-of-function mutations that result in activation of the JAK–STAT pathway, their clinical significance and correlations remain under scrutiny (Milosevic Feenstra et al. 2015). Germline *JAK2* mutations have also been described in familial thrombocytosis, as discussed earlier in the chapter.

ET subjects with *JAK2*-V617F are older, with higher haemoglobin and white cell count, and lower platelet and serum erythropoietin levels than those with *CALR* mutations (Rumi et al. 2014). The *JAK2* allele burden has not demonstrated any survival impact or transformation to leukaemia, although an increase in allele burden is associated with a higher incidence of splenomegaly, transformation to myelofibrosis and increased incidence of arterial embolic events.

Thrombopoietin Receptor

As described previously, proliferation and differentiation of *MPL* is regulated by thrombopoietin. ET subjects have a somatic gain-of-function mutation in exon 10 of *MPL*, resulting in substitution of tryptophan at codon 515 with leucine (*MPL*-W515L), lysine (*MPL*-W515K) or alanine (*MPL*-W515A) (Skoda 2009). The mutation, located within the juxtamembrane region of the protein, causes activation of the receptors in the absence of ligand, resulting in exaggerated cytokine-independent growth and hypersensitivity with overphosphorylation of the JAK2, STAT3 and STAT5 proteins. A number of additional somatic mutations have also been described in ET, including W515S, W515R, S204P, L510P, A519Y and S505N, commonly seen in familial thrombocytosis. Further mutations outside exon 10 of *MPL*, including T1191, S204F and Y591D, are all gain-of-function mutations, although their clinical significance remains unclear (Skoda 2009; Milosevic Feenstra et al. 2015)

The *MPL* mutations are found in 3–5 % of ET subjects, who are usually older with more marked thrombocytosis, lower haemoglobin and reduced bone marrow cellularity compared with triple-negative ET subjects or those harbouring a *JAK2* or *CALR* mutation (Nangalia and Green 2014). To date, there is no evidence in the literature to suggest a survival difference between subjects who harbour the *MPL* mutation and those with a *JAK2* or *CALR* mutation (Nangalia and Green 2014).

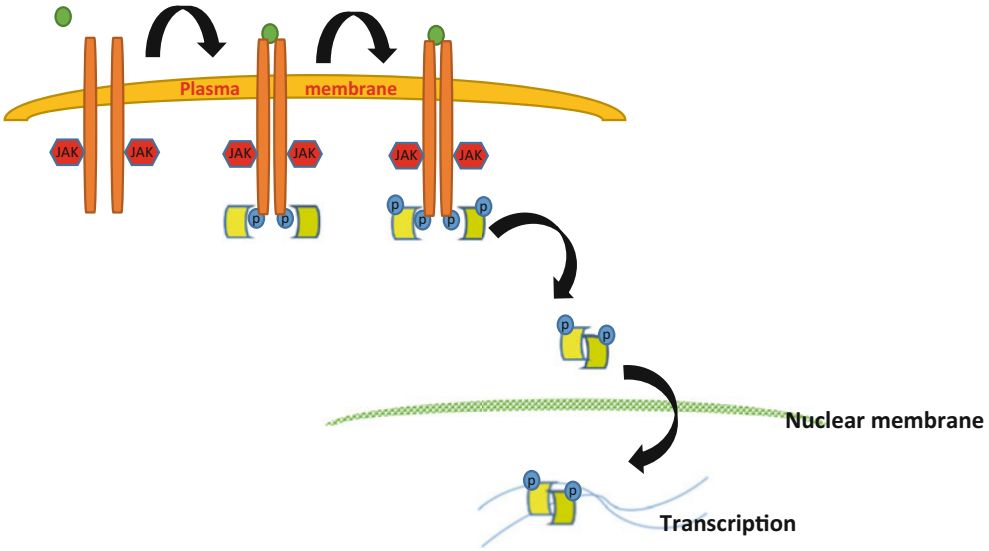

Fig. 8 Tyrosine kinase activity of the JAK pathway. Tyrosine kinase is activated when a regulatory molecule ( ) binds and the two cytokine receptor molecules ( ) come closer, bringing the JAK molecules ( ) together. This leads to phosphorylation, which further activates JAK and phosphorylates the receptors, leading to binding of STAT ( ). When STAT binds to the receptor, it is also phosphorylated by JAK. Once phosphorylated, the STATs combine to form a dimer and an active transcription factor. indicates phosphorylation

Calreticulin

This decade has seen a great shift in the understanding of MPN, with the discovery of somatic frame shift mutations at exon 9 of the *CALR* gene (Nangalia et al. 2013). CALR is an endoplasmic reticulum calcium-binding chaperone. A number of mutations within exon 9 of the *CALR* gene have been described, including type 1 (52 base pair insertion) and type 2 (5 base pair insertion) mutations; other minor mutation anomalies have also been described, such as 46 and 48 base pair deletions (Palandri et al. 2015). All mutation types result in a single base pair frameshift and alteration of the C-terminal sequence of the protein. Consequently, mutant proteins lack the usual C-terminal endoplasmic retention signal, resulting in disturbances in calcium binding (Nangalia et al. 2013). Although the mechanism of how *CALR* mutations drive the pathogenesis of ET remains unclear, JAK–STAT hyperactivation remains integral to the disease process (Rampal et al. 2014); for example, mutant *CALR* has been demonstrated to drive the JAK2–STAT pathway via activation of the thrombopoietin receptor (Chachoua et al. 2015).

CALR mutations account for approximately 30 % of cases of ET. Both variants of *CALR* mutations are associated with a higher platelet count, lower haemoglobin and lower leukocyte count than *JAK2* mutations (Rotunno et al. 2014; Rumi et al. 2014). More specifically, male gender is associated with type 1 *CALR* mutations and younger age with type 2 variants; furthermore, platelet count is significantly higher in subjects with type 2 versus type 1 *CALR* mutations. Long-term analyses have not demonstrated any differences in overall survival between ET subjects with *CALR* mutations and those with *JAK2* or *MPL* mutations.

Triple Negative

Subjects who lack the *JAK2*, *CALR* and *MPL* mutations are termed triple negative (TN); their diagnosis is through clinical and histological findings. TN subjects can have mutations outside the usual driver mutations sites or have a hereditary element to their disease (Milosevic Feenstra et al. 2015). Long-term outcome remains unclear; although subjects have fewer thromboembolic events, there is a suggestion of higher rates of blast transformation (Tefferi et al. 2014). It is interesting to note that, in this cohort, hyperactivity of the JAK–STAT pathway has been demonstrated despite the absence of a driver mutation (Rampal et al. 2014).

Epigenetic Regulators

Somatic mutations in the genes implicated in epigenetic regulation have become integral in understanding the complexity of MPNs. Regulators of DNA methylation and chromatin structure in haematopoietic progenitors are mutated in

a number of myeloid malignancies, including MPNs (Nangalia and Green 2014). Unlike mutations in *JAK2*, *CALR* and *MPL*, which are usually mutually exclusive, mutations in epigenetic regulators can co-occur with these so-called phenotypic driver mutations. Epigenetic modifiers include tet methylcytosine dioxygenase 2 (TET2), isocitrate dehydrogenase 1 (IDH1), IDH2, additional sex combs-like 1 (ASXL1), enhancer of zeste homologue 2 (EZH2) and DNA methyltransferase 3A (DNMT3A). DNA methylation has an important role in cell differentiation, as it can regulate the expression of particular genes without affecting the DNA sequence. Because it is reversible, DNA methylation has a potential role in targeted therapy (Milosevic and Kralovics 2013). The TET protein first identified less than a decade ago is responsible for the conversion of 5-methylated cytosine into the hydroxymethylated form and is mutated in 4 % of ET subjects. Although, overall, TET mutation is associated with increased risk of disease transformation, its specific effect in ET remains unknown (Tefferi et al. 2009; Nangalia and Green 2014). Mutations in IDH1/2, which catalyze conversion of isocitrate to alpha-ketoglutarate, occur in less than 1 % of ET patients. Such mutations are a predictor of overall poor survival and increased risk of transformation to leukaemia. Mutations in DNMT3A, a DNA methyltransferase, and ASXL1, an activator and inhibitor of transcription, are seen in as many as 5–8 % MPN subjects; however, both are more common in PMF (Milosevic and Kralovics 2013).The order of acquisition of such mutations is of great interest and now widely accepted to have significant clinical implications; for example, subjects in whom *JAK2* mutation is acquired first are more likely to develop PV and have higher risk of thrombosis than subjects that acquired the *TET2* mutation first (Ortmann et al. 2015) .

Risk Stratification

Risk stratification forms an important basis for discussion of prognosis with subjects and planning for initiation of therapy (see Fig. 9). It should be a tailored approach. Because of the increased thromboembolic risk associated with advancing age, subjects over the age of 60 years or with a prior thrombohaemorrhagic event fall into the high risk category, regardless of symptoms (Tefferi et al. 2001; Marchioli et al. 2005). The low risk category includes subjects under the age of 60 years, platelet count less than 1500×10^9/L and without a history of thromboembolic events. The intermediate category is compiled of subjects who are between the ages of 40 and 60 years and have no previous thromboembolic events but might possess one or more cardiovascular risk factors (Harrison et al. 2010; Barbui et al. 2011).

Activated granulocytes have a central role in endothelial injury so it is not surprising that a high white cell count is associated with an increased risk of thrombosis (Carobbio et al. 2008; Landolfi et al. 2008). This was highlighted in a number of studies, including the PT-1 study, where a white cell count within the upper limit of normal was associated with a greater incidence of thrombotic events (Campbell et al. 2012). The IPS-ET thrombosis model, currently under evaluation, further builds on this and incorporates the presence/absence of the *JAK2*-V617F mutation to predict survival (Barbui et al. 2012; Passamonti et al. 2012). The role of *JAK2* mutation in thrombotic risk is still under discussion; however, the accepted consensus is that *JAK2* mutation is associated with an increased risk of thrombosis, especially compared with subjects harbouring the *CALR* mutation (Cheung et al. 2006; Rumi et al. 2014). It is foreseeable that mutational status, including *CALR*, *MPL* and epigenetic markers, will come to play a central role in prognosis and in guiding pharmacological management.

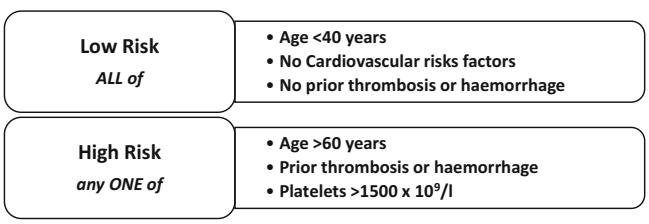

Fig. 9 Risk stratification in essential thrombocythaemia (Harrison et al. 2010)

Treatment

Risk stratification guides treatment strategies for patients with ET (Fig. 9). Antiplatelet therapy (low dose aspirin) is recommended for all patients where there are no contraindications (Harrison et al. 2010). In view of the bleeding risk in extreme thrombocytosis (platelet count $>1000 \times 10^9$/L), antiplatelet therapy should only be used with caution. Cytoreductive therapy is indicated in high-risk patients, as outlined above.

Initiation of cytoreductive therapy is dependent on risk stratification, profound constitutional symptom burden and tolerability of therapy. Subjects above the age of 60 years should be offered hydroxycarbamide, a drug that has proven efficacy in high-risk patients (Cortelazzo et al. 1995; Harrison et al. 2005). Hydroxycarbamide is well tolerated, although it continues to be associated with mouth and leg ulcerations, immunosuppression, nail changes and conflicting reports of increased risk of leukaemia. It is teratogenic and should be avoided in men and women who are of childbearing age. In older patients who are intolerant of other therapies, radioactive phosphorus and busulphan can

	<60 years	> 60 years
•**First line**	•Interferon	•Hydroxycarbamide
•**Second line**	•Anagrelide •Hydroxycarbamide	•Buslphan •Anagrelide •Pipobroman •Intrferon

Fig. 10 Summary of cytoreductive drugs used in essential thrombocythaemia

be used; however, both can increase the risk of progression to acute leukaemia over a period of long-term use.

In high-risk patients under the age of 60 years, the favoured regimens are anagrelide or interferon, or indeed hydroxycarbamide (Fig. 10). Anagrelide has demonstrated benefit in platelet control (Gisslinger et al. 2013). It is reserved for younger patients and those without cardiovascular compromise, because of the side effects of arrhythmias. Anagrelide is also associated with a risk of bone marrow fibrosis and progression to MF (Campbell et al. 2009). Interferon alpha, usually delivered in injectable form, has demonstrated efficacy in improving platelet count, constitutional symptoms and splenomegaly and in reducing JAK2 and CALR allele burden (Tefferi and Barbui 2015). It can be associated with side effects such as flu-like symptoms and mood and endocrine disturbances.

How to Approach a Case of Thrombocytosis

In the clinical setting it is important to distinguish reactive and spurious thrombocytosis from clonal thrombocytosis, as summarised in Fig. 11. A thorough history and examination can help guide initial investigations. Evidence of raised acute-phase reactants, inflammation, bleeding or recent surgery suggests that reactive thrombocytosis is a possibility, although it does not exclude an underlying MPN. Low serum ferritin is indicative of iron deficiency, which should be corrected and platelet count monitored; in addition, it is important to monitor the haematocrit because an elevation after correction of iron deficiency suggests PV as a diagnosis. The peripheral blood smear is invaluable in guiding the diagnostic pathway. If there is evidence of a leucoerythroblastic film or dysplasia, a MPN or MDS diagnosis should be sought and molecular analysis with bone marrow investigations undertaken early. If platelet count remains elevated, molecular analysis of *JAK2*-V617F, *CALR* and *MPL* are recommended; identification of a mutation does not always distinguish ET from other MPN disorders so bone marrow histology and cytogenetics are recommended, especially in the presence of abnormal peripheral blood film. If the bone marrow histology reveals fibrosis, WHO grade $\geq$1 myelofibrosis must be considered. If there is evidence of dysplasia or ring sideroblasts, myelodysplastic syndrome is another potential diagnosis. Where there is absence of mutation and MPN remains a suspected diagnosis, bone marrow histology and cytogenetics should be performed. It is important to always consider the possibility of CML, so analysis of BCR-ABL should be performed where there is strong suspicion or where diagnosis is unclear. The role of epigenetic mutation analysis in the clinical setting remains undetermined.

In subjects who have a prolonged history of thrombocytosis with a family history, hereditary thrombocytosis should be considered and investigation undertaken for the mutations outlined in Table 1.

Summary

- Thrombocytosis is frequently encountered in medical and surgical subjects; it is cytokine mediated and reversed on cessation of cause.
- Reactive thrombocytosis is not associated with the same clinical sequelae as those seen in myeloproliferative neoplasms.
- Hereditary thrombocytosis is a rare cause of familial thrombocytosis and is associated with mutations in the thrombopoietin receptor and *JAK2* gene.
- There are a number of clonal causes for thrombocytosis, including essential thrombocythaemia (ET), polycythaemia vera and myelodysplastic syndrome.
- ET is a clonal myeloproliferative neoplasm.
- Common driver mutations in ET include *JAK2*-V617F, *MPL*-W515L/K and *CALR*.
- *JAK2*-positive subjects are older, have higher platelet counts and lower haemoglobin levels and are at increased risk of thrombosis.
- *CALR*-positive subjects are younger, have lower haemoglobin levels and higher platelet counts.
- Epigenetic mutations have an increasing role in diagnosis and prognostication.

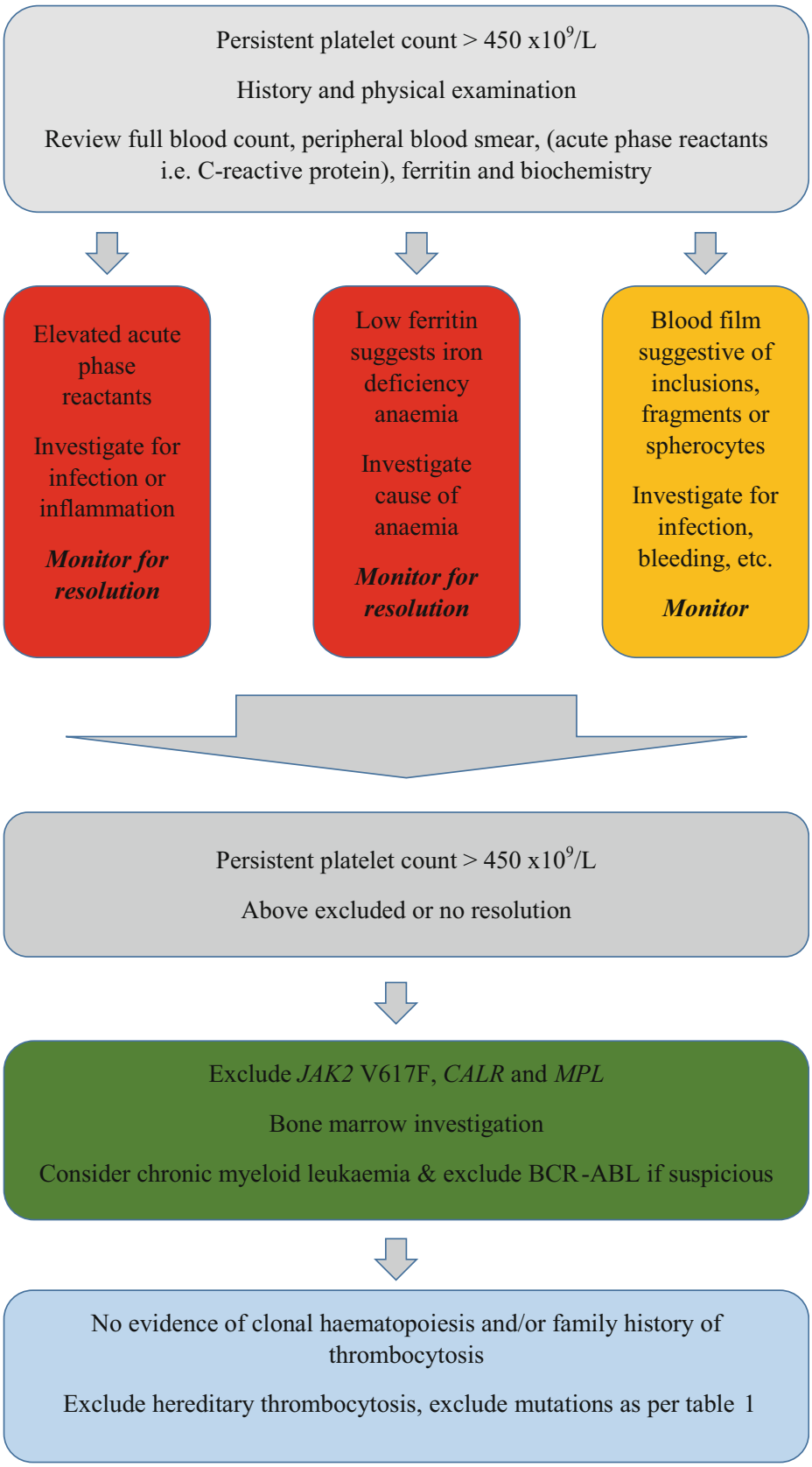

Fig. 11 Diagnostic pathway for investigating a patient with thrombocytosis

- Age over 60 years, cardiovascular risk factors, high leukocyte count and presence of the *JAK2*-V617F mutation are independent predictors of thromboembolic events.
- ET subjects over the age of 60 years or those with a previous history of thromboembolic or haemorrhagic event are classified as high risk.
- Treatment of ET is based on risk stratification. All patients, unless contraindicated, should be offered antiplatelet therapy (i.e., aspirin 75 mg daily). Cytoreductive therapy is reserved for high-risk patients or those who have gross constitutional symptoms.

Take Home Messages

- Thrombocytosis is commonly encountered in clinical practice; it is important to exclude secondary causes.
- Myeloproliferative neoplasms should be considered in patients where a secondary cause for the thrombocytosis has not been identified.
- Thrombocytosis is most commonly associated with essential thrombocythaemia but can also occur in other myeloproliferative neoplasms.
- Clonal drivers can be identified in the majority of patients with essential thrombocythaemia; JAK2-V617F in 50%, CALR in 30% and MPL in up to 5% of patients.

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Platelet Adhesive Protein Defect Disorders

Shinji Kunishima and Hirokazu Kashiwagi

Abstract

This chapter describes inherited platelet disorders linked to defects in the major platelet adhesion receptors, GPIb/IX/V and $\alpha_{IIb}\beta_3$. Bernard–Soulier syndrome is caused by abnormalities of the GPIb/IX/V complex, characterized by thrombocytopenia, giant platelets, prolonged bleeding time, and ristocetin-induced platelet agglutination. Glanzmann thrombasthenia is the result of a quantitative and/or qualitative defect in integrin $\alpha_{IIb}\beta_3$, characterized by prolonged bleeding time and absent platelet aggregation in response to multiple physiological agonists, but with normal platelet count and morphology.

Introduction

When a blood vessel is injured and bleeding occurs, platelets adhere to the exposed subendothelium, become activated and release their granule content, and form aggregates. Platelet adhesion occurs through binding of the platelet membrane glycoprotein (GP) Ib/IX/V to von Willebrand factor (VWF), which binds to the subendothelium; subsequent platelet aggregation takes place by activation of integrin $\alpha_{IIb}\beta_3$ and fibrinogen binding (Fig. 1). In addition, there are other important adhesion receptors on platelets (i.e., GPVI and α2β1) that also mediate platelet adhesion. This chapter describes platelet disorders linked to defects in the major platelet adhesion receptors. Studies have provided us with much information on the structure–function relations of receptors, but also insights into the molecular mechanisms of primary hemostasis (Table 1). The reader may refer to some excellent reviews (Gresele et al. 2015; Nurden and Nurden 2014, 2015).

Bernard–Soulier Syndrome

In 1948, Bernard and Soulier described an infant who had a severe mucocutaneous bleeding tendency with prolonged bleeding time and abnormally large platelets, but normal platelet count and clot retraction (Bernard and Soulier 1948). His sister was similarly affected and died from bleeding at a younger age. Both the parents and the other sister were asymptomatic. Similar case reports accumulated in the following years (Weiss et al. 1974; Kanska et al. 1963; Cullum et al. 1967) and the classical diagnostic criteria of autosomal recessive inheritance, thrombocytopenia, giant platelets, prolonged bleeding time, and normal clot retraction was proposed (Alagille et al. 1964; Bithell et al. 1972).

Clinical Features

Bernard–Soulier syndrome (BSS) is a very rare disorder with an estimated prevalence of one in one million (Lopez et al. 1998). However, it is likely to be more prevalent than previously believed, because patients are often misdiagnosed with immune thrombocytopenia (ITP), undiagnosed, or underreported.

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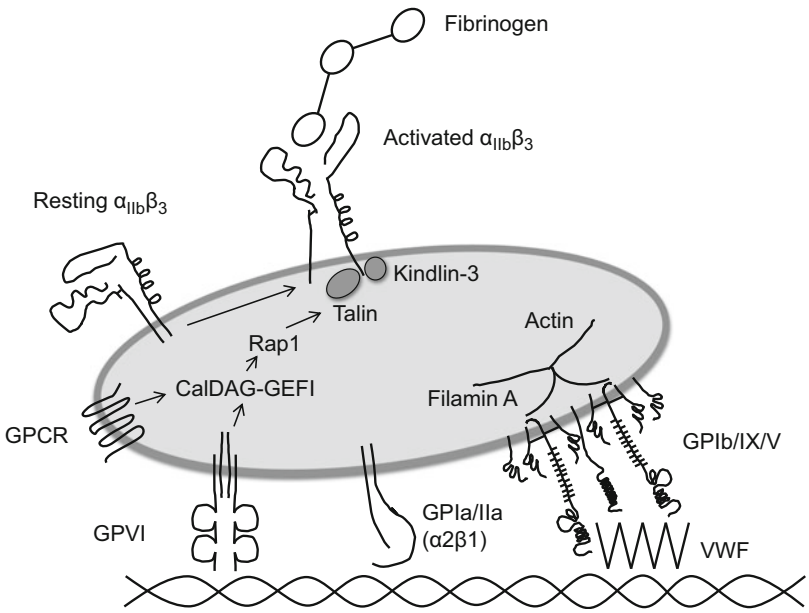

Fig. 1 Major platelet membrane glycoproteins and their ligands, with the principal inherited platelet disorders indicated

Table 1 Principal inherited platelet adhesive protein defect disorders

Disorder	Inheritance	Bleeding tendency	Platelet count	Platelet size	Molecule	Defective gene	Platelet characteristics
Bernard–Soulier syndrome	AR	Severe	Decreased	Large	GPIb/IX/V	*GP1BA*, *GP1BB*, *GP9*	Absent ristocetin-induced agglutination
Platelet-type von Willebrand disease	AD	Moderate	Normal or decreased	Large	GPIb/IX/V	*GP1BA*	Increased ristocetin-induced agglutination
Glanzmann thrombasthenia	AR	Severe	Normal	Normal	$\alpha_{IIb}\beta_3$	*ITGA2B*, *ITGB3*	Absent aggregation in response to physiological agonists
$\alpha_{IIb}\beta_3$-related macrothrombocytopenia	AD	Absent to moderate	Decreased	Large	$\alpha_{IIb}\beta_3$	*ITGA2B*, *ITGB3*	Normal to impaired aggregation
GPVI deficiency	AR	Mild	Normal	Normal	GPVI	*GP6*	Decreased response to collagen

AR autosomal recessive, *AD* autosomal dominant

In fact, in a large cohort study enrolling 131 BSS families, almost 50 % (64 of 131) of the patients had been previously misdiagnosed as having ITP and were treated with steroids and/or intravenous immunoglobulins. Of note, splenectomy was performed in 11 % of cases (14 of 131 patients) (Savoia et al. 2014). BSS is in most cases inherited in an autosomal recessive manner; only one case has been reported as autosomal dominant (Miller et al. 1992). Several founder mutations have been identified in northern Europe, Italy, Japan, and India (Kunishima et al. 2006c; Liang et al. 2005; Noda et al. 1995; Sumitha et al. 2011; Wright et al. 1993). Consanguinity is common among affected families. Even without known consanguinity, parents of a patient homozygous for a mutation often carry the same disease haplotype enriched in a certain geographical area. Among the 207 reported BSS patients, 179 (85 %) were homozygous and 28 (13 %) were compound heterozygous for mutations in one of the *GP1BA*, *GP1BB,* and *GP9* genes (Savoia et al. 2014). Patients manifest a severe bleeding tendency from early childhood, primarily from mucocutaneous tissues. Purpura, epistaxis, menorrhagia, and gingival bleeding are common but hemarthroses and deep visceral hematomas are rare. The bleeding tendency is more severe than predicted from the degree of thrombocytopenia, because platelets are dysfunctional and unable to adhere to the exposed vascular subendothelium. According to the World Health Organization (WHO) bleeding scale (grade 0, no bleeding; 1, petechial bleeding; 2, clinically significant bleeding; 3, bleeding requiring transfusion; 4, bleeding associated with fatality), more than 50 % of the patients (77 of 139 patients) had severe bleeding classified as grades 3 and 4 (Savoia et al. 2014). As is sometimes the case for other congenital bleeding disorders, a bleeding tendency is not always remarkable in

newborns or infants. Heterozygous carriers of BSS are generally asymptomatic with normal platelet function, but sometimes have mild thrombocytopenia and large platelets.

Laboratory Features

The diagnostic features are thrombocytopenia, giant platelets, prolonged bleeding time, and absent ristocetin-induced platelet agglutination. Skin bleeding times are moderately to severely prolonged (<20 min). Platelet counts in many cases are moderate to severe. The mean platelet count was 51×10^9/L (range 5×10^9–175×10^9/L) (Savoia et al. 2014). Because routine automated blood cell counting systems use the impedance method, in which blood cells are differentiated by their size and do not recognize giant platelets as platelets, they underestimate not only the platelet count but also the mean platelet volume . In this regard, optical or immunological counting is superior to conventional impedance counting (Briggs et al. 2007). Nevertheless, manual platelet counting in a calculating chamber or on peripheral blood smears is still the gold standard.

Giant platelets on peripheral blood smears are the most characteristic morphological feature of BSS. Most platelets are as large as red blood cells and the mean platelet size (determined as platelet diameter on peripheral blood smears) is twice as large as that of controls (4.8 μm, range 2.9–7.5 μm, and 2.4 μm, range 1.9–3.4 μm, respectively) (Noris et al. 2013; Savoia et al. 2014). Mean platelet volume, as measured by hematology analyzers, is also increased in BSS, giving values almost twice those of controls (Noris et al. 2013). It has been suggested that the large platelet size is a result of increased membrane deformability, which results in swelling in the plasma milieu and a tendency to readily spread on glass slides (Frojmovic et al. 1978; White et al. 1984). This is mostly caused by the defective linkage between the cytoplasmic domain of GPIbα and filamin A, leading to decreased interaction of platelet membrane and cytoskeleton. In fact, (1) giant platelets and thrombocytopenia in a mouse model of BSS can be rescued by the exogenous expression of GPIbα (Ware et al. 2000); (2) knockdown of filamin A results in production of large platelets from embryonic stem cell-derived megakaryocytes (Kanaji et al. 2012); and (3) patients with 22q11.2 microdeletion syndromes, who are haploinsufficient for the GPIbβ gene, have large platelets (Liang et al. 2007; Naqvi et al. 2011; Van Geet et al. 1998). The physical linkage between GPIb/IX/V–filamin A–actin is the major but not only determinant of platelet size. Giant platelets are also found in variants with missense mutations in the extracellular domain of GPIbα that result in the expression of dysfunctional receptors (Li et al. 1995; Miller et al. 1992; Savoia et al. 2001; Ware et al. 1993) and in type 2B von Willebrand disease (VWD) (Nurden et al. 2010; Federici et al. 2009), in which abnormal von Willebrand factor spontaneously binds to GPIb/IX/V. These findings raise the possibility that normal megakaryocytopoiesis and platelet morphogenesis are affected not only by physical interaction between GPIb/IX/V and the cytoskeleton, but also by GPIb/IX/V-mediated signal transduction. In fact, the demarcation membrane systems of megakaryocytes are irregular and vacuolar (Hourdille et al. 1990; White 1998), and proplatelet formation from megakaryocytes is defective in homozygous and heterozygous BSS (Balduini et al. 2011; Vettore et al. 2011), a mouse model of BSS (Poujol et al. 2002), and type 2B VWD (Nurden et al. 2010). Thickened marginal bands as a result of an increased number of microtubules have been observed in BSS patients and a mouse model of BSS (Balduini et al. 2009; Strassel et al. 2009).

Absence of ristocetin-induced platelet agglutination constitutes another laboratory hallmark of BSS and this abnormality is not corrected by the addition of normal plasma, a procedure the allows BSS to be distinguished from VWD. This is because the antibiotic ristocetin induces binding of VWF to GPIb/IX/V in the absence of platelet activation or high shear rates (Berndt et al. 1992); therefore, VWF fails to bind to BSS platelets deficient in GPIb/IX/V on the platelet membranes. Platelet aggregation in response to physiological agonists such as collagen, ADP, and epinephrine, as well as clot retraction, is normal. However, platelet aggregation in response to thrombin is decreased because the high affinity thrombin binding site containing GPIbα is absent and thus the capacity of α-thrombin to activate platelets through the platelet thrombin receptor PAR-1 is deficient (De Marco et al. 1994; De Candia et al. 2001). Prothrombin consumption and thrombin generation are markedly decreased because of the defective binding of factor XI and α-thrombin to platelets (Bernard and Soulier 1948; Caen and Bellucci 1983). However, functional studies of BSS platelets are difficult to perform, because of thrombocytopenia and because of the difficulty in isolating the giant platelets from red blood cells and lymphocytes. Flow cytometry-based platelet aggregation analysis has the advantage of requiring less sample for analysis (Giannini et al. 2007).

Molecular Abnormalities

Molecular abnormalities of BSS platelets were first suggested in 1969 after observing that there was a delay in the electrophoretic mobility of platelets caused by decreased sialic acid content (Grottum and Solum 1969). Later, it was found that this was caused by the lack of platelet membrane GPIb (Nurden and Caen 1975). In 1973, it was found that platelets failed to aggregate in the presence of the antibiotic

ristocetin or bovine fibrinogen (Caen and Levy-Toledano 1973; Howard et al. 1973). The platelet function abnormality was also demonstrated by the finding that platelets failed to adhere to rabbit aortic subendothelium (Weiss et al. 1974). Subsequently, GPIb (missing from BSS platelets) was identified as a platelet receptor for VWF (Hagen et al. 1980). Biochemical analysis of the surface-labeled platelet glycoproteins by two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that BSS platelets are deficient in GPIbα, GPIbβ, GPIX, and GPV (Clemetson et al. 1982; Berndt et al. 1983). However, with the exception of rare variant cases, the component subunits of the GPIb/IX/V complex are residually and heterogeneously expressed on the platelet membranes (Arai et al. 1994; Drouin et al. 1988; Kunishima et al. 1994). Flow cytometric determination of each subunit on the platelet surface is a practical and convenient method for diagnosis of BSS. The most deficient subunit in platelets is often found to indicate the genetic basis: patients with residual GPIbβ and GPIX are often associated with a *GP1BA* mutation, and those with residual GPIbα and GPIbβ are often associated with a *GP9* mutation (Kunishima et al. 2002a; Kunishima and Saito 2006). Severely decreased expression of GPIbα and GPIX suggests a *GP1BB* mutation. Flow cytometry also allows evaluation of platelet size, and double-labeling of GPIb/IX/V and $\alpha_{IIb}\beta_3$ permits comparative expression levels.

Genetic Defects

The genes encoding for GPIbα (*GP1BA*), GPIbβ (*GP1BB*), GPIX (*GP9*), and GPV (*GP5*) are located on chromosomes 17p13.2, 22q11.21, 3q21.3, and 3q29, respectively. Gene structures are similar to each other and each subunit belongs to the leucine-rich repeat (LRR) protein family (Andrews et al. 2003; Bryckaert et al. 2015; Li and Emsley 2013; Lopez 1994). After the cloning of genes encoding each of the three subunits in the late 1980s (Hickey and Roth 1993; Hickey et al. 1989; Lopez et al. 1987, 1988; Wenger et al. 1988; Yagi et al. 1994), the first causative gene defect was elucidated by Ware et al. (1990) and, so far, more than 100 different mutations have been identified (Andrews and Berndt 2013; Kunishima et al. 2002a; Lanza 2006; Lopez et al. 1998; Savoia et al. 2014; Ware et al. 1990) (Fig. 2). In vitro biosynthetic studies have shown that coordinated assembly of GPIbα, GPIbβ, and GPIX but not GPV is required for efficient surface expression of a functional GPIb/IX complex (Lopez et al. 1992, 1994; Dong et al. 1997, 1998). Thus, a defect in any of the three genes, *GP1BA*, *GP1BB*, or *GP9* by either homozygous or compound heterozygous mutation produces the BSS phenotype. No mutations have been reported in *GP5*. Mutations include missense and nonsense mutations and small insertions and deletions. Because each gene is compact and the entire open reading frame is contained within a single exon, no splice mutations were identified. This is one of the reasons why BSS is less prevalent than Glanzmann thrombasthenia (GT), in which splicing defects are common (Nurden et al. 2011a; Nurden 2006).

Genetic defects producing BSS can be mainly classified into two types: (1) common lack of expression of the GPIb/IX/V complex as a result of a biosynthetic defect that affects the synthesis, processing, or assembly of the complex and (2) less common expression of a dysfunctional complex that fails to bind ligand and affects physical linkage between the complex and the actin cytoskeleton.

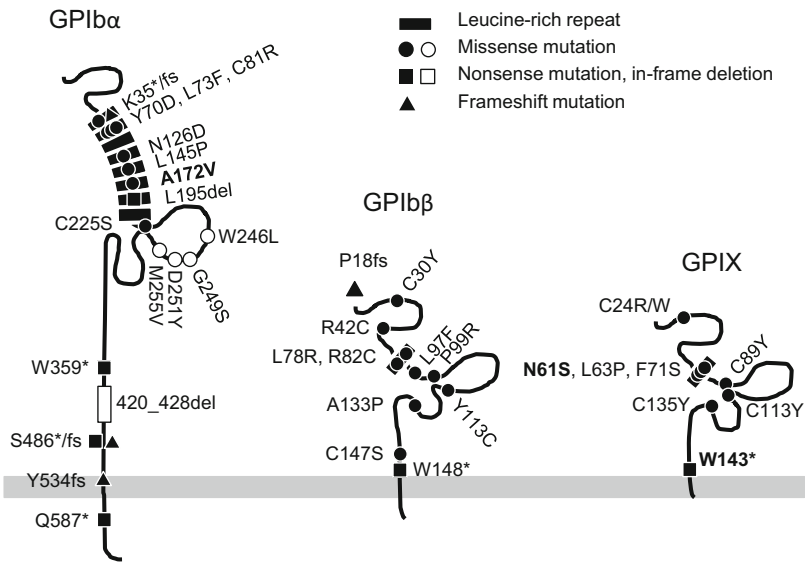

Fig. 2 GPIbα, GPIbβ, and GPIX together with mutations associated with Bernard–Soulier syndrome (*closed symbols*) and platelet-type von Willebrand disease (*open symbols*). The major founder mutations are indicated in *bold*

1. *Absent GPIb/IX/V expression*
   In BSS platelets, the expression of GPIb/IX/V is usually absent or severely decreased (Savoia et al. 2011). This is because coordinated expression of each subunit, inter-subunit interaction, disulfide association between GPIbα and GPIbβ, and interactions in the extracellular, transmembrane, and cytoplasmic domains are required for optimal assembly of the complex (Luo et al. 2007a, b; Mo et al. 2006, 2008; Luo and Li 2008). Thus, truncating mutations caused by nonsense and frameshift mutations that eliminate or alter the polypeptide sequence of a subunit not only disrupt the structural integrity of the mutated subunit but also interfere with assembly of the complex (Holmberg et al. 1997; Kunishima et al. 1994, 2002b, 2006c; Noda et al. 1995; Takata et al. 2012; Ware et al. 1990; Yamamoto et al. 2013). In some instances, truncating mutations in *GP1BA* and *GP9* are associated with residual expression of incomplete GPIbβ/GPIX and GPIbα/GPIbβ complexes, respectively. A *GP1BB* mutation is often associated with null expression of GPIbβ and severely decreased GPIbα and GPIX expression. This is because GPIbβ plays a crucial and indispensable role in expression of the complex by two GPIbβ (Cys147) molecules binding to one GPIbα (Cys500 and Cys501) to support GPIbα expression (Kunishima et al. 2006b; Lopez et al. 1994; McEwan et al. 2011; Luo et al. 2007a). A missense mutation in a subunit can lead to absent GPIb/IX/V expression through several mechanisms. Mutations at the conserved residues in the LRR motif are in many cases damaged and deteriorating (Afrasiabi et al. 2007; Clemetson et al. 1994; Dagistan and Kunishima 2007; Donner et al. 1996; Drouin et al. 2005; Kenny et al. 1998; Koskela et al. 1999; Kunishima et al. 2006a; Liang et al. 2005; Noris et al. 1997; Rand et al. 2010; Sachs et al. 2003; Sato et al. 2014; Savoia et al. 2011, 2014; Sumitha et al. 2011; Suzuki et al. 1997; Vanhoorelbeke et al. 2001; Wright et al. 1993; Zieger et al. 2009). Among these, p.Asn61Ser is the most prevalent founder mutation in northern Europe and has been reported in multiple unrelated Caucasian families (Clemetson et al. 1994; Dagistan and Kunishima 2007; Donner et al. 1996; Drouin et al. 2005; Koskela et al. 1999; Liang et al. 2005; Rand et al. 2010; Sachs et al. 2003; Savoia et al. 2011, 2014; Vanhoorelbeke et al. 2001; Wright et al. 1993; Zieger et al. 2009). In addition, mutations at the conserved cysteine residues or creating cysteines in the N- and C-terminal LRR flanking regions interfere with intramolecular disulfide bond patterns and secondary structures (Kunishima et al. 1997, 1999; Kurokawa et al. 2001; Noda et al. 1996; Simsek et al. 1994).
2. *Dysfunctional GPIb/IX/V complex*
   Some mutations in the LRR motif of GPIbα do not severely affect the stability and are associated with expression of a dysfunctional GPIb/IX/V complex. These mutations include p.Leu73Phe (Miller et al. 1992), p.Leu145Pro (Li et al. 1995), p.Ala172Val (Bolzano mutation) (De Marco et al. 1990; Ware et al. 1993), and p.Leu195del (Nancy I mutation) (de la Salle et al. 1995; Ulsemer et al. 2000) (Fig. 3).

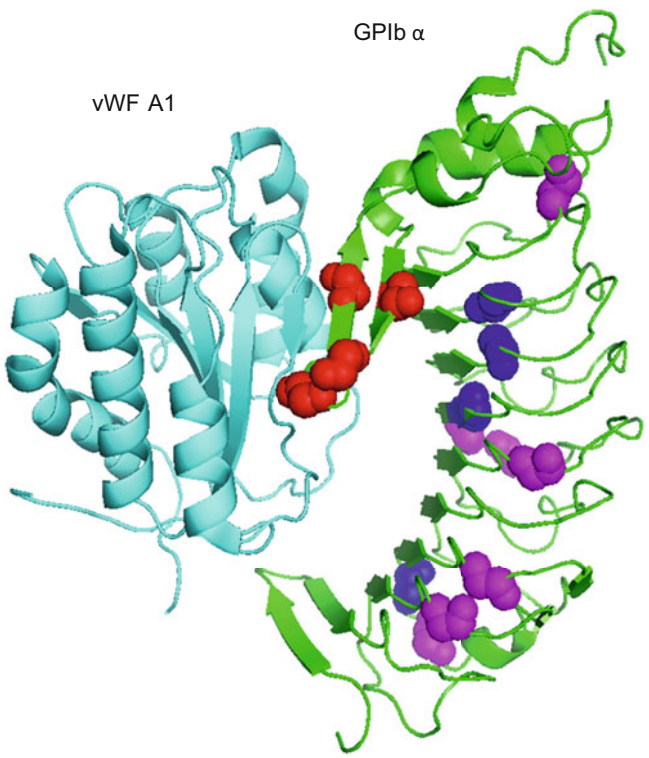

Fig. 3 Crystal structure of von Willebrand factor A1 domain–GPIbα complex (PDB 1M10). Bernard–Soulier mutations associated with absent GPIb/IX/V are shown in *magenta* and dysfunctional GPIb/IX/V are shown in *blue*. Platelet-type con Willebrand disease mutations are clustered in the β-switch region and shown in *red*

The first dysfunctional variant was described in an autosomal dominant form of BSS resulting from a heterozygous p.Leu73Phe (Miller et al. 1992). VWF binding to patients' platelets was decreased and a proteolytic fragment of GPIbα was identified on immunoblots of platelets. The p.Ala172Val mutation, also known as the Bolzano mutation, was first identified in a Italian family (De Marco et al. 1990; Ware et al. 1993). The patients' platelets had ~50 % of control levels of GPIb/IX expression. Binding of thrombin to platelets was conserved, whereas that of VWF was severely impaired. p.Ala172Val is prevalent in southern Italian populations (Savoia et al. 2001). Heterozygous patients have mild macrothrombocytopenia and may have mild bleeding symptoms; this condition was previously considered Mediterranean macrothrombocytopenia (Behrens 1975). In investigation of an Italian cohort of 216 patients with macrothrombocytopenia, 42 patients were heterozygous for p.Ala172Val, all sharing the same haplotype

(Noris et al. 2012). Patients with p.Leu145Pro (Li et al. 1995) and p.Leu195del (Nancy I mutation) (de la Salle et al. 1995; Ulsemer et al. 2000) also share similar platelet features: residual and heterogeneous expression of GPIb/IX/V and the absence of VWF binding.

Chromosome 22q11.2 Microdeletion

The 22q11.2 deletion syndrome (22q11.2DS) is one of the commonest microdeletion syndromes and encompasses DiGeorge, velocardiofacial, and conotruncal anomaly face syndromes (McDonald-McGinn and Sullivan 2011). The minimum deleted region contains *GP1BB*. Thus, patients with 22q11.2DS are obligate BSS carriers and have large platelets (Liang et al. 2007; Naqvi et al. 2011; Van Geet et al. 1998). Budarf and colleagues were the first to report BSS in a patient who had the 22q11.2DS (Budarf et al. 1995). The patient was compound heterozygous for the 22q11.2 deletion and a single point mutation that disrupts a binding site for the GATA-1 transcription factor (g.-160C>G) (Ludlow et al. 1996). This was also the first report of BSS associated with a promoter site mutation. So far, several BSS cases associated with 22q11.2DS have been described (Bartsch et al. 2011; Budarf et al. 1995; Hillmann et al. 2002; Kenny et al. 1999; Kunishima et al. 2013; Ludlow et al. 1996; Tang et al. 2004).

Acquired Bernard–Soulier Syndrome

Although very rare, acquired (or pseudo-) BSS has been described in which autoantibodies or paraproteins bind to GPIb/IX/V and impair its receptor function. It is reportedly associated with vasculitis, lymphoproliferative disorder, procainamide therapy, and Gaucher's disease (Devine et al. 1987; Kelsey et al. 1994; Stricker et al. 1985; Tornai et al. 1999). In addition, severely decreased GPIb/IX/V expression may be associated with myelodysplasia (Frigeni and Galli 2014; Berndt et al. 1988; Hicsonmez et al. 1995). In these conditions, both normal and abnormal platelet populations are present in the circulation.

Platelet-Type von Willebrand Disease

Platelet-type (or pseudo-) VWD is an autosomal-dominant bleeding disorder first described in 1982 by Weiss and colleagues (Weiss et al. 1982) and Miller and Castella (Miller and Castella 1982). Patients present mild thrombocytopenia, with or without large platelets, and a mild-to-moderate bleeding tendency (Othman and Emsley 2014; Othman et al. 2013). This disorder is characterized by an abnormally increased affinity of the platelet GPIbα for von Willebrand factor as a result of gain-of-function mutations in *GP1BA*, leading to clearance of both platelets and VWF from the circulation. Laboratory features include enhanced ristocetin-induced platelet agglutination at low concentrations of ristocetin, decreased ristocetin cofactor activity, mildly decreased VWF antigen with low VWF cofactor activity/antigen ratio, and absence of high molecular weight VWF multimers. The differential diagnosis between platelet-type VWD and type 2B VWD requires a mixing assay for ristocetin-induced platelet agglutination and genetic analysis. To date, six platelet-type VWD mutations, including five missense mutations (Enayat et al. 2012; Matsubara et al. 2003; Miller et al. 1991; Russell and Roth 1993; Woods et al. 2014) and one in frame deletion (Othman et al. 2005), have been identified. All of the missense mutations that increase the affinity of GPIbα for VWF are located at the major binding interface between the VWD A1 domain and the β-switch in the C-terminal LRR flanking region (Huizinga et al. 2002; Dumas et al. 2004; Blenner et al. 2014) (Fig. 3). The 27 base pair deletion in the GPIbα macroglycopeptide region is located outside the VWF-binding domain, suggesting allosteric modulation of VWF binding.

Glanzmann Thrombasthenia and αIIbβ3-Related Abnormalities

Glanzmann Thrombasthenia

GT is an autosomal recessive bleeding disorder caused by quantitative or qualitative abnormalities in integrin $\alpha_{IIb}\beta_3$. GT patients usually have lifelong bleeding episodes, which are typically characterized by mucocutaneous bleeding with a variable clinical presentation ranging in severity from mild bruising to fetal hemorrhages. GT was first described in 1918 by Glanzmann as "hereditary hemorrhagic thrombasthenia," which is characterized by normal platelet counts and lack of clot retraction (Stevens and Meyer 2002). In 1966, Caen and colleagues reported 15 French cases with lack of platelet aggregation and clot retraction and named the disorder "Glanzmann thrombasthenia" (Bellucci and Caen 2002). In 1972, Caen divided GT into two groups: type I GT with complete lack of platelet aggregation, low platelet fibrinogen, and a profound defect in clot retraction, and type II GT with complete lack of platelet aggregation, a substantial amount of platelet fibrinogen, and moderately defective clot retraction (Bellucci and Caen 2002). In the mid-1970s, Nurden and Caen and Phillips and coworkers demonstrated that one of the major platelet glycoproteins, which was subsequently identified as $\alpha_{IIb}\beta_3$, was absent in three cases of type I GT (Nurden and Caen 1974; Phillips

et al. 1975). In the mid-1980s, several researchers found rare variant forms of GT, in which platelet aggregation was absent despite normal expression of $\alpha_{IIb}\beta_3$ (Jung et al. 1988; Nurden et al. 1987; Tanoue et al. 1987). Although GT patients usually have normal platelet counts and morphology, several $\alpha_{IIb}\beta_3$ mutations have been found in patients with congenital macrothrombocytopenia (Nurden et al. 2011b). Moreover, congenital abnormalities in molecules closely associated with $\alpha_{IIb}\beta_3$ activation have been identified in patients with GT-like bleeding phenotypes (Nurden and Nurden 2015).

Clinical Features

GT is rare, but is the most prevalent and globally distributed congenital disorder of the platelet adhesion receptors. Although the exact number is unknown, the incidence of GT is estimated at about one in 500,000–1,000,000 individuals and is higher in ethnic groups with higher incidences of consanguinity, such as Iraqi Jews and French Gypsies (Bolton-Maggs et al. 2006). Purpura and mucosal bleeding, such as epistaxis, gingival bleeding, and menorrhagia, are common manifestations of GT (George et al. 1990; Toogeh et al. 2004). Petechiae and ecchymoses usually appear in areas of pressure or minor trauma, although spontaneous petechiae are uncommon. Epistaxis is very common and sometimes causes severe bleeding, especially in childhood. Gingival bleeding is also very common, and excessive bleeding after dental extraction may often be the first symptom shown by GT patients. Menorrhagia occurs often in female patients after menarche and could be a critical hemorrhagic problem. Gastrointestinal bleeding or hematuria can be observed, but is less common. Intracranial hemorrhages are rare and only five cases have been mentioned in the literature (Vigren et al. 2012). These bleeding tendencies are usually noticed in infancy and the majority of patients are diagnosed before the age of 5 years.

Classification

GT is defined as a congenital bleeding disorder caused by mutations in the *ITGA2B* or *ITGB3* gene, encoding αIIb or β3, respectively. GT has been classified according to the expression of $\alpha_{IIb}\beta_3$ (George et al. 1990). Severe $\alpha_{IIb}\beta_3$-deficient patients with less than 5 % of normal $\alpha_{IIb}\beta_3$ expression are classified as type I and moderate $\alpha_{IIb}\beta_3$ deficiency with 5–20 % $\alpha_{IIb}\beta_3$ expression as type II. Clot retraction is absent and platelet fibrinogen is greatly reduced in type I GT patients, whereas clot retraction and platelet fibrinogen are subnormal in type II GT. Patients with dysfunctional $\alpha_{IIb}\beta_3$ but with more than 50 % $\alpha_{IIb}\beta_3$ expression are classified as variant type.

Laboratory Findings

GT patients have normal platelet count and normal platelet morphology, markedly prolonged bleeding time, and absent or impaired clot retraction. Platelet aggregation study using a light transmission aggregometer is highly specific for the diagnosis of GT. GT platelets lack aggregation induced by all agonists except ristocetin, including ADP, collagen, epinephrine, arachidonic acid, thrombin receptor activating peptides, and thromboxane A2 mimetic. To confirm the diagnosis of GT, abnormalities in $\alpha_{IIb}\beta_3$ should be demonstrated and this is usually done by flow cytometric analysis. Quantitative analysis of surface expression of $\alpha_{IIb}\beta_3$ can be performed using a series of monoclonal antibodies against $\alpha_{IIb}\beta_3$ complex, α_{IIb}, or β_3. Dysfunction of $\alpha_{IIb}\beta_3$ can be assessed by binding of fibrinogen or ligand-mimetic antibodies, such as PAC-1. Although genetic analysis of $\alpha_{IIb}\beta_3$ is usually unnecessary for diagnosis except in the cases of variants, it can contribute to management decisions, because type I patients with a premature stop codon or truncated protein might produce alloantibodies against $\alpha_{IIb}\beta_3$ after platelet transfusion (Fiore et al. 2012; Kashiwagi et al. 2011).

Molecular Basis

The *ITGA2B* and *ITGB3* genes are located within a single 260-kilobase segment in the q21–23 band on chromosome 17. More than 200 mutations in either gene have been recorded (sinaicentral.mssm.edu/intranet/research/glanzmann) and the list is still expanding using the latest methods for genetic analysis (Buitrago et al. 2015; Nurden et al. 2015).

Large gene deletions are exceptional, although nonsense mutations, out-of-frame small deletions, and insertions that cause premature terminations of transcript are frequently identified. Many missense mutations or in-frame insertions or deletions are also described. Although the majority of mutations lead to loss of $\alpha_{IIb}\beta_3$ expression, certain mutations affect ligand binding, leading to GT variants (Fig. 4a) (Nurden et al. 2011a).

1. *Mutations leading to loss of $\alpha_{IIb}\beta_3$ expression*
   The αIIb subunit is synthesized as pro-αIIb, and pro-αIIb forms complexed with the β_3 subunit in the endoplasmic reticulum. After transfer of pro-$\alpha_{IIb}\beta_3$ complex to the Golgi apparatus, pro-αIIb is cleaved to give mature heavy and light chains of αIIb, and mature $\alpha_{IIb}\beta_3$ is moved to the platelet surface. If proper complex formation fails, residual α_{IIb} or β_3 undergoes proteasomal degradation (Duperray et al. 1989). Therefore, defects in $\alpha_{IIb}\beta_3$ expression are caused not only by nonsense or out-of-frame mutations but also by missense or in-frame mutations that affect proper $\alpha_{IIb}\beta_3$ complex formation or cellular trafficking. Although these missense or in-frame mutations are located in whole coding regions of the *ITGA2B* and *ITGB3* genes, the β-propeller domain of α_{IIb} and the β-I domain of β_3 are apparently hot spots

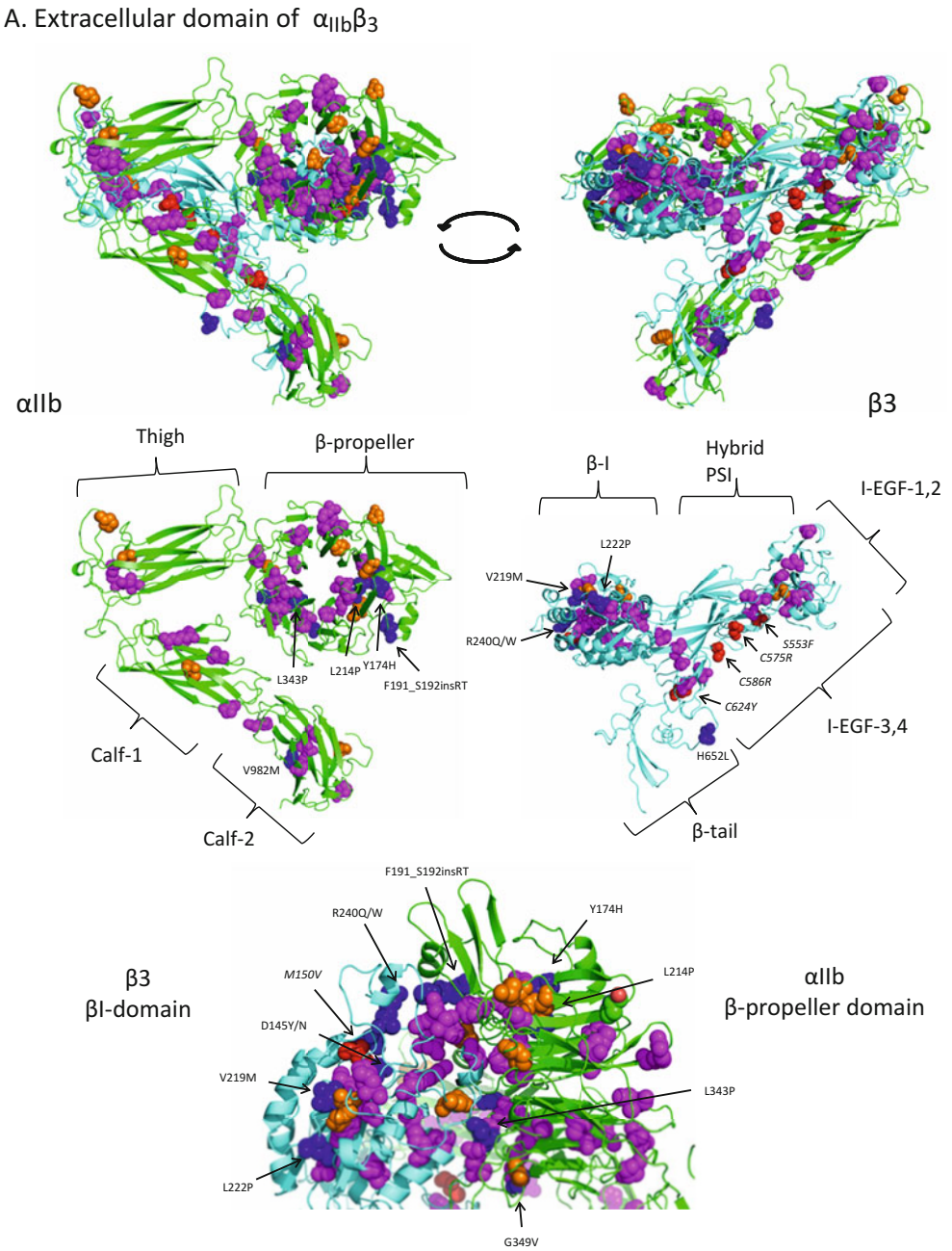

Fig. 4 Crystal structure of the extracellular domain (**a**) and transmembrane and cytoplasmic domains (**b**) of $\alpha_{IIb}\beta_3$. Amino acid substitutions identified in GT or $\alpha_{IIb}\beta_3$-associated macrothrombocytopenia are indicated. Mutations causing loss-of-expression, loss-of function, and gain-of-function are indicated as *magenta*, *blue*, and *red spheres*, respectively. *Orange spheres* indicate mutations with unknown effects. The gain-of-function mutations are also indicated in *italic*. (**c**) Gene structure and positions of mutations identified in the *FERMT3* gene

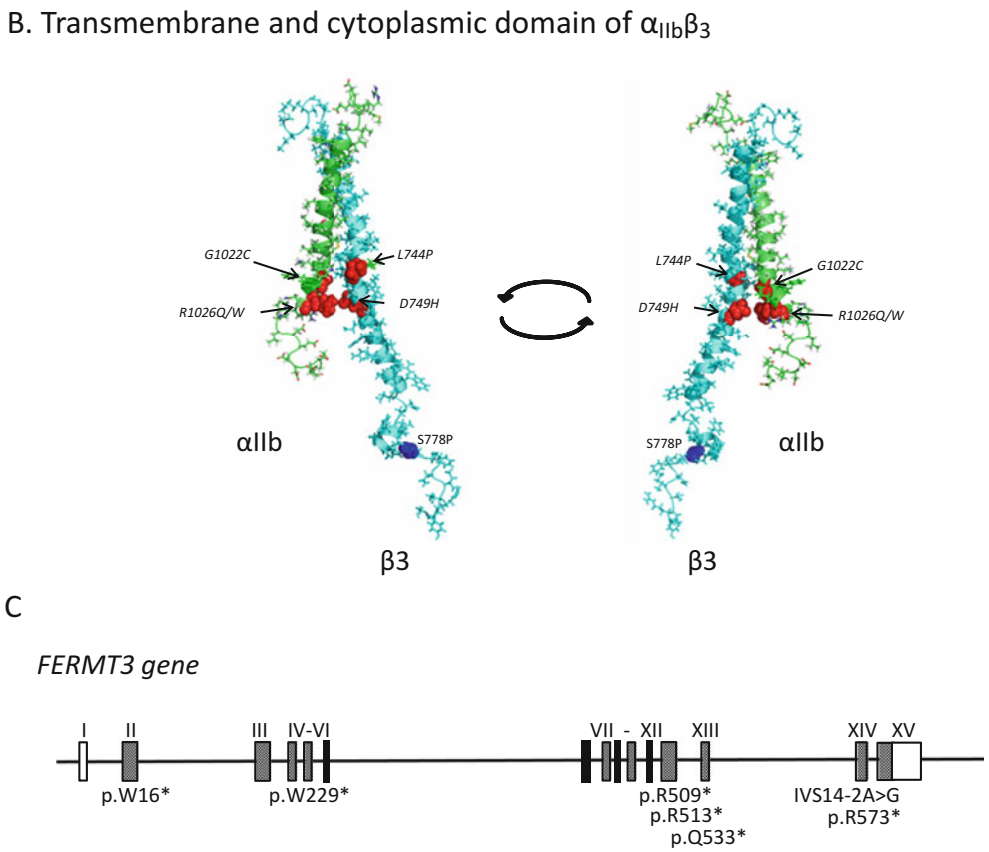

Fig. 4 (continued)

for the mutations, indicating that proper conformation and interaction of these regions is crucial for normal $\alpha_{IIb}\beta_3$ expression (Buitrago et al. 2015; Nurden et al. 2015).

2. *Loss-of-function mutations*
The β-propeller domain of αIIb and the β-I domain of β3 form a ligand-binding site and most GT variants are caused by mutations in these regions. The β-propeller domain of αIIb consists of seven four-stranded β-sheets (W1–W7) arranged in a torus around a pseudosymmetry axis (Xiao et al. 2004). The crucial regions of the αIIb for ligand binding are located within the 2-3 loop and the 4-1 loop of W3. The mutations p.Tyr174His, p.Phe191_Ser192insArgThr, and p.Leu214Pro in these regions lead to loss of ligand binding (Honda et al. 1998; Kiyoi et al. 2003). Crystal structure analysis also shows that key residues for ligand binding are located on the metal ion-dependent adhesion site (MIDAS) and the ADMIDAS (adjacent to MIDAS) domain in the β-I domain of β3 (Springer et al. 2008). Mutations in these regions, such as p.Asp145Tyr and p.Arg240Gln/Trp, make $\alpha_{IIb}\beta_3$ unstable by increasing its sensitivity to divalent cation chelation and abolishing ligand binding (Nurden et al. 2011a). Binding of talin and kindlin-3 to the β3 cytoplasmic tail is the final step of $\alpha_{IIb}\beta_3$ activation of "inside-out" signaling (Moser et al. 2008; Tadokoro et al. 2003). Three mutations leading to GT variants by impaired inside-out signaling have been found in the β3 cytoplasmic tail: p.Ser778Pro, p.Arg750*, and a splicing mutation leading to frameshift after Arg768 (Chen et al. 1992; Wang et al. 1997; Hauschner et al. 2012). Moreover, interaction between the membrane proximal to the Calf-2 domain of αIIb and the I-EGF4/β-tail domain of β3 is important for propagation of conformational signals from cytoplasmic tails. Mutations affecting ligand binding have also been identified in these regions (Kamata et al. 2005).

3. *Gain-of-function mutations*
Mutations leading to constitutive active conformation of $\alpha_{IIb}\beta_3$ have been identified in several GT patients. Cysteine residues in the I-EGF domain of β3 are a hot spot for these gain-of-function mutations, indicating the importance of the sulfide bridge in this region in maintaining the resting bent form of $\alpha_{IIb}\beta_3$. These mutations, such as p.Cys575Arg, mostly cause severe impaired surface expression of $\alpha_{IIb}\beta_3$, which could account for the bleeding

tendency (Mor-Cohen et al. 2007). However, in the case of homozygous p.Cys586Arg, platelets express ~20 % of constitutively activated $\alpha_{IIb}\beta_3$ on the platelet surface and increase fibrinogen binding (Ruiz et al. 2001). β3 p.Cys586Arg-transduced mice showed high mortality caused by bleeding, indicating that constitutive binding of fibrinogen to platelets leads to impaired platelet function and causes bleeding tendency instead of thrombosis (Fang et al. 2013).

αIIbβ3-Related Macrothrombocytopenia

Although a p.Arg1026Gln mutation in the αIIb cytoplasmic tail was reported in a GT-like patient with mild thrombocytopenia and anisocytosis (Hardisty et al. 1992; Peyruchaud et al. 1998), it was thought that mutations in the *ITGA2B* and *ITGB3* genes do not affect platelet counts and morphology until Ghevaert and colleagues found the p.Asp749His mutation in the β3 cytoplasmic tail in a family with congenital macrothrombocytopenia (Ghevaert et al. 2008). Subsequently, αIIb p.Arg1026Trp was identified in five patients from three independent Japanese families with macrothrombocytopenia (Kunishima et al. 2011). Interestingly, the salt bridge between αIIb Arg1026 and β3 Asp749 is essential for maintaining the resting state of $\alpha_{IIb}\beta_3$ (Hughes et al. 1996); disruption of the salt bridge by the mutations leads to constitutive activation of $\alpha_{IIb}\beta_3$. Several mutations in *ITGA2B* and *ITGB3* have now been identified in patients with congenital macrothrombocytopenia (Gresele et al. 2009; Nurden et al. 2011b; Kashiwagi et al. 2013). Mostly, these mutations are located in the juxtamembrane regions of αIIb or β3 and lead to constitutive $\alpha_{IIb}\beta_3$ activation with aberrant "outside-in" signaling, which may cause abnormal proplatelet formation (Kashiwagi et al. 2013; Kunishima et al. 2011; Bury et al. 2012; Kobayashi et al. 2013) (Fig. 4b).

Patients heterozygous for these mutations show mild thrombocytopenia with large platelets. Surface expression of $\alpha_{IIb}\beta_3$ is mildly to moderately decreased, but platelet aggregation is usually normal and bleeding tendency is absent or minimal. In the case of a compound heterozygote with p.Gly1022Cys and p.Arg453* in αIIb, surface expression of $\alpha_{IIb}\beta_3$ was decreased to 3–11 % with relatively severe macrothrombocytopenia (22×10^9–102×10^9/L). In the case of a compound heterozygote with αIIb p.Arg1026Gln and a 13 base pair intronic deletion (c.1440-13_1440-1del) leading to abnormal splicing of exon 15 in the *ITGA2B* gene, surface expression of $\alpha_{IIb}\beta_3$ was decreased to 12–20 % with modest thrombocytopenia (100×10^9–160×10^9/L) (Nurden et al. 2011b). We generated αIIb p.Arg1026Trp knock-in mice and showed that $\alpha_{IIb}\beta_3$ expression of homozygous knock-in platelets decreased to less than 5 % with mild macrothrombocytopenia (Kiyomizu et al. 2014). These results indicate that subjects homozygous for these activating mutations or compound heterozygous associated with $\alpha_{IIb}\beta_3$-null alleles may show a GT-like phenotype with macrothrombocytopenia. Reduced surface expression of $\alpha_{IIb}\beta_3$ appears to be the main cause of bleeding tendency, and thrombocytopenia and functional abnormalities in $\alpha_{IIb}\beta_3$ can exacerbate the symptoms.

GT-Like Variants Caused by Abnormalities in Kindlin-3 or CalDAG-GEFI

Kindlin-3 is an essential molecule for activation of β1, β2, and β3 integrins in platelets and leukocytes. Mutations of kindlin-3 coded by *FERMT3* cause a GT-like severe bleeding phenotype with immunodeficiency, named leukocyte adhesion disease (LAD)-III (Moser et al. 2008; Malinin et al. 2010). To date, at least seven mutations leading to premature termination of translation have been identified in *FERMT3* (Fig. 4c) (Crazzolara et al. 2015; Kuijpers et al. 2009; Malinin et al. 2009; Mory et al. 2008; Svensson et al. 2009). CalDAG-GEFI is a crucial signaling molecule for $\alpha_{IIb}\beta_3$ activation through activation of the small GTPase Rap1, which is an essential step for the binding of talin to the β3 cytoplasmic tail (Crittenden et al. 2004). Two mutations in CalDAG-GEFI coded by *RASGRP2*, p.Gly248Trp and p.Phe497fs*22, were found by exome sequencing of families suffering severe bleeding with impaired platelet aggregation response (Nurden and Nurden 2015; Canault et al. 2014). Expression defect mutations, p.Leu360del and p.Lys309*, were also identified in a Japanese girl with life-long bleeding tendency (Kato et al. 2015). Interestingly, CalDAG-GEFI defective platelets showed reduced but substantial aggregation response to high concentrations of agonists such as ADP and collagen; PMA-induced aggregation was normal. Susceptibility to infection was not reported in patients with CalDAG-GEFI abnormalities, in contrast to LAD-III (Canault et al. 2014; Kato et al. 2015).

Acquired Glanzmann Thrombasthenia

Acquired GT is a rare hemorrhagic disorder caused by autoantibodies, alloantibodies, or paraproteins that impair ligand binding to $\alpha_{IIb}\beta_3$. About 30 cases have been described so far, and they are often in association with lymphoproliferative or autoimmune disorders (Giannini et al. 2008; Porcelijn et al. 2008). In acquired GT, despite normal expression of $\alpha_{IIb}\beta_3$, platelet aggregation with agonists

(apart from ristocetin) is impaired, as in GT variants. Although immune thrombocytopenia (ITP) is the major concomitant, thrombocytopenia is usually mild or absent, probably as a result of a subclass of the antibodies (IgG2 or IgG4) or treatment such as splenectomy (Porcelijn et al. 2008).

Deficiency of Collagen Receptors

α2β1 (GPIaIIa) and GPVI are two major collagen receptors on platelets, and deficiency of these receptors, mostly GPVI deficiency, causes mild mucocutaneous bleeding tendency with a defective platelet aggregation response to collagen.

GPVI is a 62-kDa transmembrane protein and consists of two immunoglobulin-like domains (D1 and D2), a short mucin-like domain, a transmembrane domain, and a cytoplasmic domain. GPVI is noncovalently associated with the FcRγ chain, and ligand binding to the GPVI/FcRγ complex induces inside-out signals via the Syk/SLP-76/PLCγ2 pathway (Zahid et al. 2012). GPVI deficiency is a rare platelet disorder with mild bleeding tendency (Table 2). The first case of GPVI deficiency was reported in 1987, was associated with ITP, and showed anti-GPVI antibodies in the plasma (Sugiyama et al. 1987). Most reported subjects with acquired GPVI deficiency are female, concomitant with ITP or systemic lupus erythematosus (SLE), and have anti-GPVI antibodies either in plasma or platelet eluates (Table 2). Antibody-mediated GPVI depletion was first confirmed in mice injected with an anti-GPVI monoclonal antibody, leading to loss of GPVI expression in circulating platelets for at least 2 weeks after injection (Nieswandt et al. 2001). These antibodies cause metalloproteinase-mediated ectodomain cleavage (Andrews et al. 2007) or immune-induced internalization and degradation (Takayama et al. 2008), leading to loss of surface expression of GPVI. Congenital GPVI deficiencies caused by mutations in the *GP6* gene were firstly identified in 2009, and two missense mutations and three out-of-frame mutations have been reported to date (Fig. 5a). The p.Arg58Cys mutation in D1 abrogate collagen binding and the p.Ser195Asn mutation in D2 mainly lead to reduced

Table 2 Clinical profiles of patients with GPVI deficiency

	Age	Concomitant	Platelet count ($\times 10^9$/L)	Bleeding tendency	Autoantibody	Mutation in *GP6*	References
Deficiency							
Female	26		285	Purpura, epistaxis, menorrhagia			Moroi et al. (1989)
Male	26		100–130	Purpura, epistaxis			Ryo et al. (1992)
Acquired GPVI deficiency							
Female	58	ITP	13–140	Purpura, massive epistaxis required blood transfusion	+		Sugiyama et al. (1987)
Female	47	SLE	20–303	Purpura	+		Takahashi and Moroi (2001)
Female	25	ITP	105–135	Purpura	+		Boylan et al. (2004)
Female	20	ITP	48	Purpura	+		Kojima et al. (2006), Akiyama et al. (2009)
Female	55	ITP	2–167	Purpura, epistaxis	+		Gardiner et al. (2008)
Female	12	ITP	80–150	Purpura, epistaxis	+		Akiyama et al. (2009)
Female	22	SLE	4–441	Bleeding diathesis	+		Nurden et al. (2009)
Congenital GPVI deficiency							
Female	56		110–140	Pupura, epistaxis, postcoital bleeding, postoperative bleeding			Arai et al. (1995)
Female	55	Gray platelet syndrome	18–35	Epistaxis, menorrhagia, postsurgery and gastric bleeding			Nurden et al. (2004)
Female	10		280	Epistaxis		p.R58C c.356_360dup	Dumont et al. (2009)
Female	31		208	Purpura, epistaxis, menorrhagia, postsurgery and posttraumatic bleeding		p.S195N c.142_157del	Hermans et al. (2009)
Female	22		Normal	Purpura		c.711_712insA	Matus et al. (2013)
Female	11		Normal	Purpura		c.711_712insA	Matus et al. (2013)
Male	23		Normal	Purpura		c.711_712insA	Matus et al. (2013)
Male	12		Normal	Purpura		c.711_712insA	Matus et al. (2013)
Female	5		Normal	Asymptomatic		c.711_712insA	Matus et al. (2013)

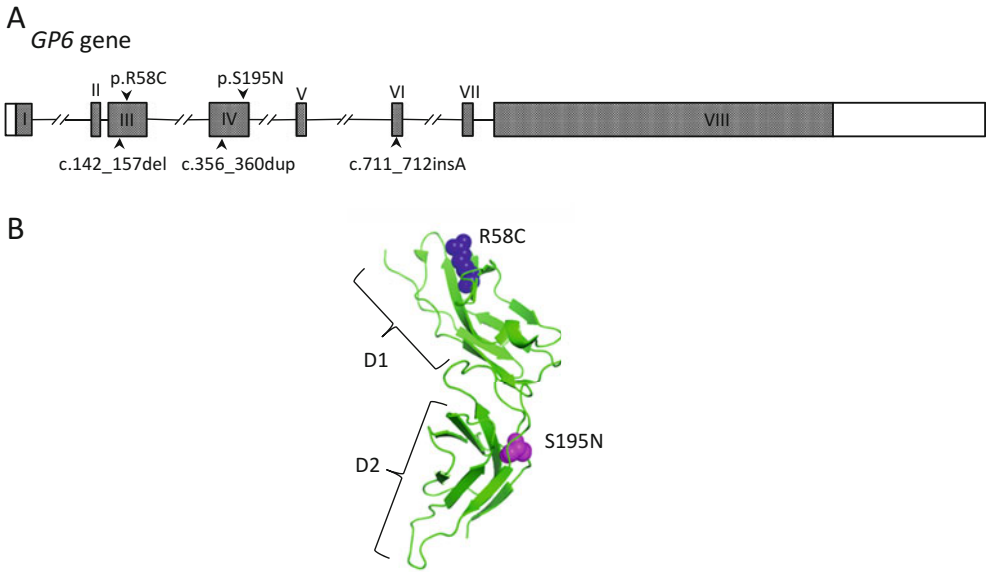

Fig. 5 (**a**) Gene structure and positions of mutations identified in the *GP6* gene. (**b**) Crystal structure of the two immunoglobulin-like domains of GPVI (D1 and D2) and positions of missense mutations. *Magenta* and *blue spheres* indicate lose-of-expression and loss-of-function mutations, respectively

expression of GPVI (Dumont et al. 2009; Hermans et al. 2009) (Fig. 5b). Five GPVI-deficient patients homozygous for an adenine insertion in exon 6 of *GP6* were reported in four unrelated families in Chile (Matus et al. 2013).

Two patients with impaired collagen-induced platelet aggregation caused by α2β1 deficiency have been described (Kehrel et al. 1988; Nieuwenhuis et al. 1985). However, the specific pathology of the reduced expression of α2β1 has not been elucidated.

Take Home Messages

- Bernard–Soulier syndrome and Glanzmann thrombasthenia are the most clinically significant platelet function disorders.
- Recent investigations have provided much information on the structure–function relations of GPIb/IX/V and $\alpha_{IIb}\beta_3$, and insights into the molecular mechanisms of primary hemostasis.
- Novel pathological conditions caused by defects in GPIb/IX/V and $\alpha_{IIb}\beta_3$ have been elucidated.

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Defects of Soluble Agonists

Marco Cattaneo

Abstract

Congenital abnormalities of platelet receptors for soluble agonists represent one of the major categories of congenital defects of platelet function. Despite the fact that a selective pathway of platelet activation is abnormal, the most common abnormalities of these receptors involve the two major pathways of amplification of platelet aggregation and secretion: the adenosine diphosphate (ADP) and the thromboxane A2 (TxA2) pathways. For this reason, the abnormalities of platelet function that can be observed using traditional laboratory diagnostic tests are mostly unspecific. In order to raise the correct diagnostic hypothesis, it is important to focus on specific abnormalities: (1) defective platelet aggregation induced by ADP, even at very high concentrations, identifies congenital abnormalities of the platelet $P2Y_{12}$ receptor; (2) abnormal platelet aggregation and secretion induced by a thromboxane A2 mimetic identifies abnormalities of the thromboxane prostanoid (TP) receptor. Confirmation of the diagnostic hypothesis rests on specific tests and the identification of molecular abnormalities of the encoding genes.

Classification of Congenital Disorders of Platelet Function

Congenital disorders of platelet function are generally classified based on the functions or responses that are abnormal. However, since platelet functions are intimately related, a clear distinction between disorders of platelet adhesion, activation, secretion, aggregation, and procoagulant activity is in many instances problematic. For this reason, in 2003 I proposed a classification of congenital disorders of platelet function based on abnormalities of platelet components that share common characteristics (Cattaneo 2003): (1) platelet receptors for adhesive proteins, (2) platelet receptors for soluble agonists, (3) platelet granules, (4) signal transduction pathways, and (5) procoagulant phospholipids. Inherited disorders of platelet function that are less well characterized will be grouped into a sixth category of miscellaneous disorders. This classification approach is summarized in Table 1.

In this chapter, congenital abnormalities of platelet receptors for soluble agonists will be reviewed.

Defects of the Platelet P2 Receptors

Human platelets express at least three distinct receptors stimulated by adenosine nucleotides: $P2Y_1$; $P2Y_{12}$, which bind adenosine diphosphate (ADP); and $P2X_1$, which binds adenosine triphosphate (ATP) (Cattaneo 2011).

ADP plays a key role in platelet function because, when it is secreted from the platelet dense granules where it is stored, it amplifies the platelet responses induced by other platelet agonists (Cattaneo 2011; Packham and Mustard 2005), stabilizes platelet aggregates (Cattaneo et al. 1990; Trumel et al. 1999), and inhibits the antiplatelet effects of prostacyclin (Cattaneo and Lecchi 2007). The interaction of

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Table 1 Classification of congenital disorders of platelet function

1. Abnormalities of the platelet receptors for adhesive proteins
• GPIb-IX-V complex: Bernard-Soulier syndrome, platelet-type von Willebrand disease
• GPIIb-IIIa ($\alpha_{IIb}\beta_3$): Glanzmann thrombasthenia
• GPIa-IIa ($\alpha_2\beta_1$)
• GPVI
2. Abnormalities of the platelet receptors for soluble agonists
• $P2Y_{12}$ receptor
• Thromboxane prostanoid receptor
• α_2-Adrenergic receptor
• PAR-1 defect[a]
3. Abnormalities of the platelet granules
• δ-Granules: non-syndromic δ-storage pool deficiency, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, MRP4 deficiency, thrombocytopenia with absent radius syndrome, Wiskott-Aldrich syndrome
• α-Granules: gray platelet syndrome, Quebec platelet disorder, 11q terminal deletion disorder, white platelet syndrome, Medich platelet disorder, X-linked macrothrombocytopenia with thalassemia, arthrogryposis renal dysfunction and cholestasis (ARC) syndrome
• α- and δ-Granules: α,δ-storage pool deficiency
4. Defects of signal transduction
• Abnormalities of the arachidonic acid/thromboxane A_2 pathway: defects in phospholipase A_2, cyclooxygenase, thromboxane synthetase
• Abnormalities of GTP binding proteins: Gαq deficiency, Gαi1 defect, hyperresponsiveness of platelet Gsα
• Defects in phospholipase C activation: partial selective PLC-β2 isozyme deficiency
• Abnormalities in transcription factors
• Abnormality in GPVI/FcRc signaling leukocyte adhesion deficiency-III (LAD-III)
• CalDAG/GEFI defect
5. Abnormalities of membrane phospholipids
• Scott syndrome
• Stormorken syndrome
6. Miscellaneous abnormalities of platelet function
• Primary secretion defects
• Other (osteogenesis imperfecta, Ehlers-Danlos syndrome, Marfan syndrome, hexokinase deficiency, glucose-6-phosphate deficiency)

[a]Defect of PAR-1 function, associated with a polymorphism of the encoding gene, was identified in a patient, who also displayed an inherited defect of the $P2Y_{12}$ receptor for ADP (Patel et al. 2014), and in patients with the gray platelet syndrome (De Candia et al. 2007; Larocca et al. 2015). The table is an updated version of a previously published one (Cattaneo 2003)

ADP with its G_q-coupled, $P2Y_1$ receptor mediates a transient rise in cytoplasmic Ca^{2+}, platelet shape change, and rapidly reversible aggregation, while its interaction with the G_i-coupled, $P2Y_{12}$ receptor mediates inhibition of adenylyl cyclase, phosphatidylinositol 3-kinase (PI3-K) activation, and the amplification of the platelet aggregation response (Cattaneo 2011; Cattaneo and Gachet 1999; Gachet et al. 1995). About 70 % of the platelet binding sites for ADP are associated with $P2Y_1$, while the remaining 30 % are associated with $P2Y_{12}$ (Cattaneo 2011).Concomitant activation of both the G_q and G_i pathways by ADP is necessary to elicit normal aggregation (Cattaneo 2011; Cattaneo and Gachet 1999; Gachet et al. 1995).

ATP, being an antagonist of both $P2Y_1$ and $P2Y_{12}$, inhibits platelet activation by ADP (Mustard and Packham 1970; MacFarlane and Mills 1975); however, through its interaction with $P2X_1$, it can also activate platelets by inducing a very rapid influx of Ca^{2+} from the extracellular medium, which is associated with a transient platelet shape change (Rolf et al. 2001). Platelet activation by ATP amplifies the platelet responses to other agonists, especially in flow conditions that are characterized by high shear stress (Cattaneo et al. 1994a; 1994b; 2002a, 2003a; Oury et al. 2001, 2004; Hechler et al. 2003).

Although a patient with $P2Y_1$ deficiency was described in abstract form by in 1999 (Oury et al. 1999), no further details of this family have been published in a full article since this 1999 abstract. A $P2Y_1$ gene dimorphism, 1622AG, was associated with a significant effect on platelet ADP response, with a greater response in carriers of the G allele (frequency 0.15). The response to all tested concentrations of ADP in GG homozygotes was higher than in AA homozygotes, but greatest with 0.1 μM ADP (on average, 130 % higher) (Hetherington et al. 2005).

The same authors also described a patient with a severe bleeding diathesis associated with a naturally occurring dominant negative $P2X_1$ mutant, lacking one leucine within a stretch of four leucine residues in the second transmembrane domain (amino acids 351–354) (Oury et al. 2000). However, the patient also displayed a severe defect of

ADP-induced platelet aggregation that cannot be explained by the defect of $P2X_1$ and could by itself account for the bleeding diathesis of the patient. Therefore, the relationship between genotype and phenotype in this patient remains unclear.

Defects of the Platelet ADP Receptor $P2Y_{12}$

$P2Y_{12}$ Deficiency

Congenital $P2Y_{12}$ deficiency is an autosomal recessive disorder. The first patient with severe $P2Y_{12}$ deficiency was described in 1992 (Cattaneo et al. 1992). He had a lifelong history of excessive bleeding, prolonged bleeding time (15–20 min), reversible aggregation in response to weak agonists, and impaired aggregation in response to low concentrations of collagen or thrombin. However, the most typical feature was that ADP, even at very high concentrations (>10 μM), did not induce full and irreversible platelet aggregation. When added to citrate platelet-rich plasma (PRP) of the patient, ADP induced normal platelet shape change followed by normal, brisk increase of light transmission, indicative of platelet aggregation; however, when the extent of platelet aggregation was about 10 % of maximal, platelet aggregates rapidly reversed to baseline. This is a typical feature of congenital $P2Y_{12}$ deficiency, which was observed also in patients who were subsequently described (Cattaneo 2011).

This typical abnormality of ADP-induced aggregation of $P2Y_{12}$-deficient platelets can be explained in light of the recently elucidated regulation of platelet RAP1 activation (Stefanini et al. 2015). RAP1 activation is controlled by the antagonistic balance between the calcium-sensitive RAP guanine nucleotide exchange factor (GEF), CalDAG-GEFI, which stimulates GTP loading on RAP1 (GTP-bound, active form), and the RAP GTPase-activating protein (GAP), RASA3, which catalyzes GTP hydrolysis (ADP-bound, inactive form). Platelet stimulation through ITAM-coupled and G protein-coupled (which include the ADP receptor $P2Y_1$) receptors leads to the activation of phospholipase C (PLC) isoforms, which convert phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate (IP3). IP3 then mediates a rise in the cytosolic Ca^{2+} concentration, which triggers CalDAG-GEFI-dependent RAP1 activation. CalDAG-GEFI signaling eventually subsides and RASA3 inactivates RAP1. In order to maintain RAP1 in the activated, ATP-bound form, RASA3 must be inhibited: this effect depends on phosphoinositide 3-kinase signaling, which in platelets is under the control of Gαi-coupled receptors, such as $P2Y_{12}$ and the α2A receptor for epinephrine (Stefanini et al. 2015). Therefore, deficiency of $P2Y_{12}$ prevents the inactivation of RASA3, thus explaining why ADP/$P2Y_1$-induced platelet aggregates rapidly deaggregate, independently of the concentration of ADP used.

Other abnormalities of platelet function observed in the first patient with congenital $P2Y_{12}$ deficiency, which were confirmed also in most similar patients who had been subsequently described, were (1) no inhibition by ADP of prostaglandin (PG) E_1-stimulated platelet adenylyl cyclase, but normal inhibition by epinephrine; (2) normal shape change and borderline-normal mobilization of cytoplasmic Ca^{2+} induced by ADP; and (3) presence of approximately 30 % of the normal number of binding sites for [^{33}P]2MeSADP on fresh platelets (Cattaneo et al. 1992) or [^{3}H]ADP on formalin-fixed platelets, which are associated with the ADP receptor $P2Y_1$ (Gachet et al. 1995). Five additional patients with severe $P2Y_{12}$ deficiency, belonging to four kindreds, were subsequently described: all of them displayed a platelet phenotype that was similar to that of the first patient (Nurden et al. 1995; Dawood et al. 2009).

The study of the son of a patient with homozygous $P2Y_{12}$ deficiency allowed the characterization of heterozygous $P2Y_{12}$ deficiency (Cattaneo et al. 2000): ADP-induced platelet aggregation was reversible for ADP concentrations ≤10 μM, but was full and irreversible for concentrations of ADP ≥10 μM; the inhibition of PGE_1-induced increase in platelet cyclic AMP was impaired, albeit not completely absent; the number of platelet binding sites for [^{33}P]2MeS-ADP was intermediate between his mother's and normal subjects'; finally, the platelet secretion was impaired. Because the secretion defect in this patient's platelets was not associated with impaired production of TXA_2 or low concentrations of platelet granule contents, it is very similar to that described in patients with an ill-defined and probably heterogeneous group of congenital defect of platelet secretion, sometimes referred to with the general term "primary secretion defect" (Cattaneo 2003; Cattaneo et al. 1997).

Mutation Analysis in $P2Y_{12}$ Deficiency

The $P2Y_{12}$ gene of two patients, including the first described one, displayed homozygous base pair deletions in the open reading frame, resulting in frameshifts and premature truncation of the protein (Table 2) (Dawood et al. 2009; Conley et al. 2001). Molecular analysis of the $P2Y_{12}$ gene of two sisters revealed an identical single bp deletion (378delC) occurring just beyond the coding sequence for the third transmembrane domain in $P2Y_{12}$, resulting in a frame shift (p.Thr126fs) and premature truncation of the protein (Conley et al. 2001). As only alleles encoding the mutated DNA sequence were found by PCR analysis, the patients were considered homozygous for the 378delC mutation. However, a subsequent study revealed that they suffer

Table 2 Characteristics of the patients with congenital $P2Y_{12}$ deficiency

Patient number [reference]	$P2Y_{12}$ mutations	$P2Y_{12}$-associated binding sites	Platelet aggregation induced by high concentrations of ADP	Bleeding time (min)	History of pathological bleeding
1 (Cattaneo et al. 1992)	p.[Gln98fs] +[Gln98fs]	Severely reduced	Reduced and reversible	15; 20	Yes
2 (Nurden et al. 1995)	p.[Phe240fs]+[?]	Severely reduced	Reduced and reversible	>20	Yes
3 (Cattaneo et al. 2000)	p.[0]+p.Thr126fs	Severely reduced	Reduced and reversible	>30	Yes
4 (Cattaneo et al. 2000)	p.[0]+p.Thr126fs	Severely reduced	Reduced and reversible	20	Yes
5 (Shiraga et al. 2005)	p.[0]+[0]	NA	Reduced and reversible	>15	Yes
6 (Dawood et al. 2009)	p.[Gly12fs] +[Gly12fs]	NA	Reduced and reversible	NA	Yes
7 (Nurden et al. 1995; Hollopeter et al. 2001)	p.[Phe240fs]+[=]	Intermediate	Full and irreversible	NA	No
8 (Cattaneo et al. 2000; Fontana et al. 2009)	p.[0]+[=]	Intermediate	Full and irreversible	13	No

Patient 7 is the daughter of patient 2; patient 8 is the son of patient 3. The upper normal limit of the bleeding time varies between 8 and 10 min in different laboratories
NA not available

from $P2Y_{12}$ deficiency owing to haploinsufficiency and to the 378delC mutation in their remaining allele (Table 2) (Fontana et al. 2009). A patient of Japanese origin was homozygous for a single nucleotide substitution in the transduction initiation codon (ATG to AGG) (Table 2) (Shiraga et al. 2005). The molecular defect that is responsible for the severe $P2Y_{12}$ deficiency in another patient (Cattaneo et al. 2000) is less well defined (Hollopeter et al. 2001). One mutant allele contains a deletion of 2 bp within the coding region, at codon 240, resulting in a frameshift and early truncation of the protein. Surprisingly, the other allele did not display any mutation. The findings that the patient's platelets contained $P2Y_{12}$ transcripts derived from the mutant allele only and that his daughter, who had a heterozygous phenotype, inherited the mutant allele from her father and a normal allele from her mother suggest that the propositus has an additional, as yet unknown, mutation that silences the other allele (Table 2).

Congenital Dysfunction of $P2Y_{12}$

One patient with congenital bleeding disorder associated with abnormal $P2Y_{12}$-mediated platelet responses to ADP, whose platelets display normal number of dysfunctional $P2Y_{12}$ receptors, has been described (Cattaneo et al. 2003b). Platelets from this patient displayed reduced and reversible aggregation in response to 4 μM ADP, similar to normal platelets with a blocked $P2Y_{12}$ receptor. However, the response to 20 μM ADP, albeit still decreased and reversible, was more pronounced and was further inhibited by a $P2Y_{12}$ antagonist, indicating residual receptor function. ADP failed to lower adenylyl cyclase activity stimulated by PGE_1 in the patient's platelets. Analysis of the patient's $P2Y_{12}$ gene revealed, in one allele, a G to A transition changing the codon for Arg256 in transmembrane (TM) 6 to Gln and, in the other, a C to T transition changing the codon for Arg265 in extracellular loop (EL) 3 to Trp (Table 3). Neither mutation interfered with receptor surface expression but both altered receptor function, since ADP inhibited the forskolin-induced increase of cyclic AMP markedly less in cells transfected with mutant $P2Y_{12}$ than in wild-type cells (Cattaneo et al. 2003b). These observations, in accordance with previous studies of the $P2Y_1$ receptor (Moro et al. 1998, 1999) and the recent solution of the receptor structure (Zhang et al. 2014a, b), helped to identify regions in TM6 and EL3, whose structural integrity is necessary for normal receptor function. A heterozygous point mutation in the same region of the molecule, which changed codon 258 coding for proline (CCT) to threonine (ACT) (p.Pro258Thr), was described in a patient with mild bleeding disorder and severely impaired ADP-induced platelet aggregation (Table 3) (Remijn et al. 2007). Since the proline to threonine substitution alters the protein hydrophobicity, size, and rotational mobility, it is likely to affect the function of $P2Y_{12}$.

A heterozygous mutation, predicting a lysine to glutamate (p.Lys174Glu) substitution in $P2Y_{12}$, was identified in one patient with mild type 1 von Willebrand disease (VWD) (Daly et al. 2009). Platelets from this patient showed reduced and reversible aggregation in response to ADP, up to 10 μM. The reduced response was associated with an approximate 50 % reduction in binding of [^{3}H]2MeS-ADP. Considering that Lys174 is situated in the second extracellular loop of $P2Y_{12}$, adjacent to Cys175, which may be important for the expression of the ADP binding site receptor, and that a hemagglutinin-tagged p.Lys174Glu $P2Y_{12}$ variant showed surface expression in Chinese hamster ovary cells, it is likely that the Lys174Glu mutation is responsible for disruption of the ADP binding site of the receptor.

Table 3 Characteristics of the patients with congenital dysfunction of $P2Y_{12}$

Patient number [reference]	$P2Y_{12}$ mutations	$P2Y_{12}$-associated binding sites	Platelet aggregation induced by high concentrations of ADP	Bleeding time (min)	History of pathological bleeding
9 (Cattaneo et al. 2003b)	p.[Arg256Gln] +[Arg265Trp]	Normal	Reduced and reversible	19; 20	Yes
10 (Cattaneo et al. 2003b)	p.[Arg265Trp]+[=]	Normal	Full and irreversible	7	No
11 (Cattaneo et al. 2003b)	p.[Arg265Trp]+[=]	Normal	Full and irreversible	6.5	No
12 (Remijn et al. 2007)	p.[Pro258Thr]+[=]	NA	Reduced and reversible	Normal/slightly prolonged	Yes
13 (Daly et al. 2009)	p.[Lys174Glu]+[=]	Reduced	Reduced and reversible	NA	Yes[a,b]
14 (Nisar et al. 2011)	p.[Pro341Ala]+[=]	Reduced	Normal	NA	Yes (mild)
15 (Patel et al. 2014)	p.[Arg122Cys] +[Arg122Cys]	Reduced (low affinity)	Reduced and reversible	NA	Yes[c]
16 (Lecchi et al. 2015)	p.[His187Gln]+ [His187Gln]	Normal (low affinity)	Reduced and reversible	NA	Yes
17 (Lecchi et al. 2015)	p.[His187Gln]+ [His187Gln	Normal (low affinity)	Reduced and reversible	NA	Yes

Patients 10 and 11 are the son and the daughter of patient 9. The upper normal limit of the bleeding time varies between 8 and 10 min in different laboratories

NA not available

[a]Patient 13 also had type 1 von Willebrand disease

[b]The data refer to the mother of the propositus, who had the same defect of her son, but had normal von Willebrand factor plasma levels. The propositus had type 1 von Willebrand disease, which accounted for a bleeding history of moderate severity

[c]The patients' platelets also displayed reduced platelet response to thrombin and PAR-1, associated with the presence of an intronic polymorphism of F2R gene, encoding for PAR-1, which had been shown to be associated with reduced PAR-1 receptor activity

It is interesting to note that, for reasons that are presently unclear, the two patients with heterozygous dysfunctional $P2Y_{12}$ described above (one with p.Pro258Thr and one with p.Lys174Glu) display a much more severe impairment of ADP-induced platelet aggregation compared to the two patients who are heterozygous for $P2Y_{12}$ deficiency (Remijn et al. 2007; Daly et al. 2009) and to the two children of patient AC, who are heterozygous for the p.Arg265Gln mutation (Cattaneo et al. 2003b) (Table 1).

$P2Y_{12}$ undergoes internalization and subsequent recycling to maintain receptor responsiveness, which is regulated by four amino acids at the extreme C-terminus of this receptor, a putative postsynaptic density 95/disc large/zonula occludens-1 (PDZ)-binding motif (Nisar et al. 2011). A heterozygous mutation in the PDZ-binding sequence of $P2Y_{12}$ (p.Pro341Ala) was detected in a patient with reduced expression of $P2Y_{12}$ on the cell surface, which was associated with significantly compromised $P2Y_{12}$ (Nisar et al. 2011).

A woman with lifelong bleeding history, characterized by both spontaneous and excessive postsurgical and post-traumatic hemorrhages, displayed severely impaired ADP-induced platelet aggregation and inhibition of PGE1-induced VASP phosphorylation, suggesting severely impaired $P2Y_{12}$ receptor function. Binding studies revealed a decreased expression of the receptor and low affinity for 2MeSADP (Patel et al. 2014). Thrombin- or protease receptor-activated peptide-1 (PAR-1)-induced platelet aggregation was impaired, compared to control; the addition of the $P2Y_{12}$ antagonist cangrelor did not reproduce the defect in control platelets, suggesting that it was not attributable to the lack of amplification by released ADP interacting with $P2Y_{12}$. The patient was found to be homozygous for c364C>T transition and p.Arg122Cys mutation. Arg122 is within the highly conserved region DRY motif, occurring at the boundary between transmembrane III and extracellular loop II, which is associated with G protein-coupled receptor activation and/or G protein coupling and plays a role in regulating receptor conformational states (Rovati and Capra 2014). The patient also displayed an intronic polymorphism in the F2R gene, which encodes PAR-1, which had been shown to be associated with reduced PAR-1 receptor activity. Studies in transfected cell lines revealed that the reduced p.Arg122Cys $P2Y_{12}$ expression is a consequence of agonist-independent internalization followed by subsequent receptor trafficking to lysosomes (Patel et al. 2014).Two sisters of the proposita had the same phenotype and genotype, while her two sons had mild bleeding symptoms and were heterozygous for the defect.

More recently, two brothers of Turkish descent, with bleeding disorder and severe defect of $P2Y_{12}$-mediated

platelet responses to ADP, were described (Lecchi et al. 2015), whose platelets displayed normal number of binding sites for the $P2Y_{12}$ antagonist [^{3}H]PSB-0413 (Ohlmann et al. 2013). However, the affinity of [^{3}H]PSB-0413, and that of the agonists ADP and 2MeSADP, was reduced. Patients' DNA showed a homozygous c.847T>A substitution that changed the codon for His-187 to Gln (p.His187Gln). Crystallographic data and molecular modeling studies indicated that His187 in transmembrane (TM)5 is important for agonist and nucleotide antagonist binding and located in a region undergoing conformational changes (Zhang et al. 2014a, b; Lecchi et al. 2015).

Bleeding Diathesis

Patients with defects of $P2Y_{12}$ experience mucocutaneous bleedings and excessive postsurgical or post-traumatic blood loss. The severity of their ISTH bleeding score (Ohlmann et al. 2013) of two sisters with the same molecular defect (Cattaneo et al. 2000) was quite different: 7 (mild bleeding history) and 13 (moderate/severe bleeding history) (unpublished observations). After extensive investigation of hemostasis parameters, which included measurement of the activity of clotting and fibrinolytic factors and the search for known polymorphisms of hemostasis proteins, we found no explanation for the discrepancy in the severity of bleeding manifestations in the two sisters.

The bleeding score of patients with heterozygous defect, son of one of the two sisters mentioned above, was normal; however, it must be noted that this young boy had not yet experienced situations that could challenge the hemostatic system at the time of our investigation. His bleeding time, despite the mild defect of $P2Y_{12}$, was prolonged (13 min).

Diagnosis and Treatment

The diagnosis of a $P2Y_{12}$ defect should be suspected when ADP, even at relatively high concentrations ($\geq$10 μM), is unable to induce full, irreversible platelet aggregation, while inducing normal shape change. Tests that evaluate the degree of inhibition of adenylyl cyclase by ADP, by measuring either the platelet levels of cyclic adenosine monophosphate (cAMP) or the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) (Zighetti et al. 2010) after the exposure of platelets to prostaglandin (PG) E_1, should be used to confirm the diagnosis. Measurement of the number of platelet binding sites for radiolabeled 2-methyl-thyo ADP or of the specific $P2Y_{12}$ antagonist [(3) H]PSB-0413 (Ohlmann et al. 2013) could be used as a further confirmatory tests, which, however, could give normal results in patients with dysfunctional $P2Y_{12}$ (Table 4).

The intravenous infusion of the vasopressin analogue desmopressin (0.3 μg/kg) shortened the prolonged bleeding time of a patient from 20 min to 8.5 min (Cattaneo et al. 1994b). After the infusion of desmopressin, which was repeated twice at 24-h intervals, the patient underwent a surgical intervention for disc hernia repair, which was not complicated by excessive bleeding. Although the efficacy of desmopressin in reducing bleeding complications of patients with defects of primary hemostasis is anecdotal (Cattaneo et al. 2002b), its administration is generally without serious side effects.

Polymorphisms of the $P2Y_{12}$ Gene

Four polymorphisms of the $P2Y_{12}$ gene were identified, which were in total linkage disequilibrium, determining haplotypes H1 and H2, with respective allelic frequencies of 0.86 and 0.14. H2 haplotype is a gain-of-function haplotype, associated with increased ADP-induced platelet aggregation (Fontana et al. 2003a). The H2 haplotype was more frequent among 184 patients with peripheral artery disease than in 330 age-matched control subjects (OR, 2.3; CI, 1.4 to 3.9; $p = 0.002$ after adjustment for diabetes, smoking, hypertension, hypercholesterolemia, and other selected platelet receptor gene polymorphisms) (Fontana et al. 2003b).

Several studies tested the hypothesis that common polymorphisms of the $P2Y_{12}$ gene might interfere with the antiplatelet effects of drugs inhibiting the $P2Y_{12}$ receptor, accounting for the well-known individual variability in the response to these agents (Cattaneo 2004, 2012). The vast majority of these studies, with few exceptions, reported that $P2Y_{12}$ polymorphisms are not associated with altered platelet function inhibition by $P2Y_{12}$ antagonists (von Beckerath et al. 2005; Angiolillo et al. 2005).

Table 4 Diagnosis of defects of the platelet $P2Y_{12}$ receptors

1. Diagnostic suspicion
– Evidence that ADP, even at high concentrations (>10 μM), is unable to induce full and irreversible platelet aggregation (light transmission aggregometry)
2. Confirmatory tests
– Evidence that ADP fails to inhibit the increase in platelet cyclic AMP concentration induced by PGE1 (or other molecules that stimulate adenylyl cyclase)
– Reduced platelet reactivity index at the VASP-phosphorylation assay
3. Supplementary test
– Defective platelet binding of agonists (e.g., radiolabeled 2MeSADP) or antagonists (e.g., radiolabeled PSB-0413) at the $P2Y_{12}$ receptor

Defects of the Platelet Thromboxane Prostanoid Receptors

The thromboxane prostanoid (TP) receptor is a G protein-coupled receptor that is encoded in humans by the TBXA2R gene. Thromboxane A_2 (TXA_2) and its endoperoxide precursors prostaglandin (PG)G_2 and PGH_2 mediate a range of physiological and pathological responses in platelets, monocytes, macrophages, endothelial cells (EC), and smooth muscle cells (SMC), which all express TP receptor (Capra et al. 2014).

Activation of the platelet TP receptor triggers platelet activation, secretion, and aggregation, which play important roles in the formation of both hemostatic plugs and pathological thrombi.

Defects of the platelet TP receptor are associated with mild-moderate impairment of primary hemostasis, which is generally characterized by pathological mucocutaneous bleeding. The phenotype of patients with TP receptor defects is similar to that described in transgenic mice lacking TP receptor (Thomas et al. 1998).

In 1981, three reports of impaired platelet responses to TXA_2 in patients with bleeding disorders were published (Lages et al. 1981; Samama et al. 1981; Wu et al. 1981). 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, the stable TXA_2 mimetic U46619 was tested and found to be unable to elicit normal platelet responses (Wu et al. 1981), 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 (Fuse et al. 1993). Binding studies of radiolabeled TXA_2 agonists and antagonists revealed that the patient platelets had normal number of TXA_2 binding sites and normal equilibrium dissociation rate constants. Despite the normal number of TP receptors, TXA_2-induced inositol 1,4,5-triphosphate formation, Ca^{2+} mobilization, and guanosine-5′-triphosphate (GTP)ase activity were abnormal, suggesting that the abnormality of these platelets was impaired coupling between TP, G protein, and phospholipase C (PLC). The platelet aggregation and secretion responses to several agonists were impaired. A similar patient, who was also affected by polycythemia vera, had previously been described by Ushikubi et al. (1987).

These two last patients were subsequently found to have an Arg60 to Leu mutation in the first cytoplasmic loop of the TxA_2R (Hirata et al. 1994), affecting both isoforms of the receptor (Hirata et al. 1996; Okuma et al. 1996). 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_2R suppresses the wild-type receptor-mediated platelet aggregation and secretion by a mechanism independent of inhibition of PLC activation (Fuse et al. 1996; Higuchi et al. 1999).

In the year 2010, a heterozygous Asp304Asn substitution in TXA_2R was described in a 14-year-old boy experiencing severe nose bleeding. Platelet aggregation and ATP secretion induced by U46619 were reduced. The TXA2R antagonist [^{3}H]-SQ29548 showed an approximate 50 % decrease in binding to the patient's platelets, indicating that the mutation is associated with reduced ligand binding (Mumford et al. 2010).

Kamae et al. (2011) described a severe defect of U46619-induced platelet aggregation and secretion in a 7-year-old girl and her father. Immunoblots showed that the TP receptor expression levels in their platelets were approximately 50 % of controls, and nucleotide sequence analysis revealed that they were heterozygous for a novel mutation, c.167dupG in the TP receptor cDNA. Expression studies in CHO cells indicated that the mutation was responsible for the expression defect in the TP receptor. Finally, Mumford et al. (2013) described a patient with reduced platelet aggregation and secretion responses to TP receptor activators, and a heterozygous Trp29 to Cys substitution. Total TP receptor expression in patient's platelets was normal, but there was reduced maximum binding and reduced affinity of binding to the TP receptor antagonist [^{3}H]-SQ29548.

Defects of the α_2-Adrenergic Receptors

In two families whose members had impaired platelet aggregation and secretion in response to epinephrine but normal responses to other agonists, the defect was a decreased number of platelet α_2-adrenergic receptors (Rao 2003; Tamponi et al. 1987). Surprisingly, in one family inhibition of platelet adenylyl cyclase by epinephrine was normal (Rao 2003). Considering that impaired platelet responses to epinephrine are observed in a relatively high percentage of otherwise normal subjects, the relationship between the described defects and bleeding manifestations still needs to be clarified.

Defects of the PAR-1 Receptors

PAR-1, encoded by the F2R gene, is one of the two GPCR for thrombin expressed by human platelets, the second one being PAR-4 (De Candia et al. 2007). Thrombin cleaves the N-terminal extracellular domain of the receptor, which exposes a new N-terminus that, by refolding, acts as ligand to the receptor. A synthetic dodecapeptide simulates the physiological ligand and is available for studies of platelet function involving PAR-1.

Isolated, congenital defects of the PAR-1 receptor for thrombin have not been described yet.

As already mentioned, a polymorphism of the encoding gene, associated with decreased receptor function, has been described in association with dysfunctional $P2Y_{12}$ in a kindred with bleeding diathesis (Patel et al. 2014).

It has been shown that some patients with the gray platelet syndrome display a decreased expression of PAR-1 on the platelet membrane, which may contribute to their platelet function defect and bleeding tendency (De Candia et al. 2007; Larocca et al. 2015).

Take-Home Message

- Abnormalities of the platelet $P2Y_{12}$ receptor for ADP and of the TP receptor for thromboxane A2 are the best characterized disorders of platelet function associated with defects of receptors for soluble agonists.
- In spite of the selective abnormality of the platelet activation pathway, general alterations of platelet aggregation and secretion are observed in affected individuals, because both secreted ADP and thromboxane A_2 play important roles in the amplification of platelet responses to other agonists.
- The inability of ADP, even at very high concentrations, to elicit full, irreversible platelet aggregation should raise the hypothesis of $P2Y_{12}$ defect.
- The inability of thromboxane A_2 analogues to cause platelet shape change and/or aggregation should raise the hypothesis of TP receptor defect.

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Defects of Platelet Granules and Secretion

Barbara Zieger

Abstract

Inherited platelet disorders are usually classified according to the defect in the platelets. These comprise defects of platelet receptors, signal transduction, cytoskeleton, and platelet granule secretion. Platelet α-granules, δ-granules, or both may be affected resulting in phenotypical changes. According to the disorder platelet size and morphology can also be altered.

Platelet Granules

Platelets contain three groups of secretory organelles: α-granules, δ-granules ("dense bodies"), and lysosomes. The role played by platelet lysosomes remains unclear.

In unactivated platelets α-granules and δ-granules are distributed throughout the whole platelet. The actin skeleton lies between them acting as a barrier. However, after platelet activation, the actin skeleton facilitates granule secretion by disassembling its network and subsequently participating in new interactions. Platelet activation leads to platelet shape change and to centralization of the granules within the platelets. Granules fuse with other granules and then with the open canalicular system (OCS), so that the granules' contents can be discharged into the extracellular space (Stenberg et al. 1984). Individual granules can, however, fuse with the platelet plasma membrane and thus discharge their contents into the extracellular space (Morgenstern et al. 1987). The fusions between the plasma membrane, granule membrane, and OCS are regulated by so-called SNARE proteins (Feng et al. 2002). These proteins are in turn influenced by other proteins (i.e., NSF or Sec1/Munc) (Lo et al. 2005). Actomyosin contraction supports granule secretion (Suzuki et al. 1999), which then intensifies platelet activation. As the granule membrane fuses with the platelet membrane during secretion, the surface of the platelets increases, as does the number of its receptors, e.g., the expression of P-selectin (α-granules) rises on the platelet surface (Fig. 1).

α-Granules

α-Granules are the most frequently appearing granules in platelets (about 40–80 α-granules per platelet measuring 200–500 nm). Interestingly, megakaryocytes and platelets can, via endocytosis, absorb fibrinogen and factor V from the extracellular space, storing them in α-granules (Bouchard et al. 2008). α-Granules contain membrane-bound proteins that are expressed on the platelet surface after platelet activation (i.e., P-selectin) and as well dissolvable proteins that are released into the extracellular space (e.g., VWF, thrombospondin, FV, fibrinogen).

P-Selectin

P-selectin is a key platelet adhesion receptor. The most important ligand for P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on almost all leukocytes. P-selectin is a typical membrane protein in the α-granule family and is thus employed as a marker when investigating an α-granule defect via flow cytometry. It is increasingly expressed on the platelet surface after platelet activation and granule secretion.

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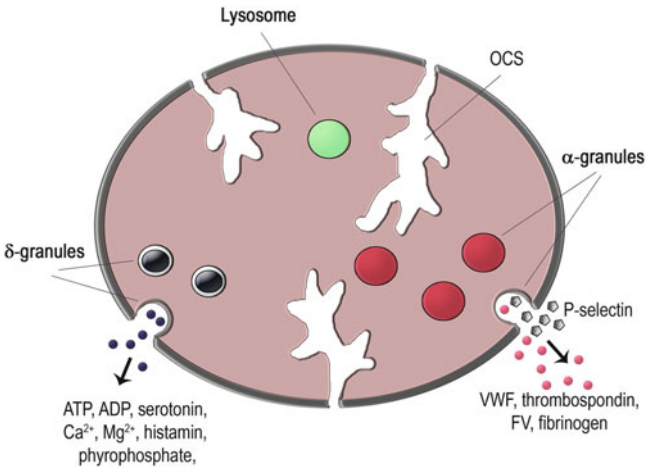

Fig. 1 Platelets contain three groups of secretory organelles: α-granules, dense (δ-) granules, and lysosomes. The contents of these granules can be mobilized for extracellular degranulation

δ-Granules ("Dense Bodies")

δ-Granules are in the family of lysosome-related organelles to which melanosomes, cytotoxic T-cell granules, and basophilic and azurophilic granules also belong. δ-Granules are smaller (approx. 200 nm) and more seldom found in platelets (3–9 δ-granules per platelet) as α-granules (Sixma et al. 1989). δ-Granules contain ATP, ADP, serotonin, histamine, pyrophosphate, and Ca^{2+}- and Mg^{2+}-ions. Serotonin and CD63 function as markers for δ-granules and their secretion. CD63 (granulophysine, LAMP3) is a typical membrane protein in lysosome-related organelles.

Platelet Granule Defects

Platelet granule defects can be detected in isolation or in conjunction with various syndromal disorders. Platelet granule defects are categorized as α-storage pool defects (α-granule secretion is reduced), δ-storage pool defects (δ-granule secretion is inadequate), and α-/δ-storage pool defects (both α- and δ-granule secretion are pathological) (Table 1).

Table 1 Genetic mutations and patterns of inheritance associated with granule defects

	Disease	Gene mutation	Inheritance
α-Granule defects	Gray platelet syndrome	*NBEAL2*	Autosomal recessive
		GFI1B	Autosomal dominant
	Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome	*VPS33B*	Autosomal recessive
	Quebec platelet defect	*PLAU*	Autosomal dominant
	X-chromosomally inherited macrothrombocytopenia	*GATA1*	X-linked dominant
	Hermansky-Pudlak syndrome	*HPS1-HPS10*	Autosomal recessive
δ-Granule defects	Chediak-Higashi syndrome	*LYST*	Autosomal recessive
	Griscelli syndrome	*RAB27A*, *MYO5A*, *MLPH*	Autosomal recessive
	TAR (thrombocytopenia with absent radius) syndrome	*RBM8A*	Autosomal recessive/ dominant
	Wiskott-Aldrich syndrome	*WAS*	X-linked recessive
α-/δ-Granule defects	RUNX1 defect	*RUNX1*	Autosomal dominant
	Paris-Trousseau syndrome/Jacobsen syndrome	*FLI1*	

α-Granule Defects

Gray Platelet Syndrome

Gray platelet syndrome (GPS) is characterized by a grayish appearance of the patient's platelets in the peripheral blood smear and the presence of a platelet α-granule defect. The platelets appear grayish due to a severe shortage (or even absence) of α-granules (Raccuglia 1971; White and Brunning 2004). Platelet vacuoles are often visible. These patients usually present with macrothrombocytopenia. The α-granule secretion defect can be diagnosed via flow cytometry analysis (CD62 expression). The severity of bleeding tends to vary enormously from individual to individual. The patients can develop bone marrow fibrosis and splenomegaly. This is an autosomal recessive inherited disease caused by a mutation in the *NBEAL2* (neurobeachin-like 2) gene which codes for neurobeachin-like 2 protein (Kahr et al. 2001; Gunay-Aygun et al. 2011; Albers et al. 2011). NBEAL seems to be involved in granule trafficking and the development of α-granules.

Mutations in the *GFI1B* gene (which encodes for the transcriptional repressor growth factor independent 1B) lead to a truncated protein and are associated with an autosomal dominant form of GPS. In these patients macrothrombocytopenia with dyserythropoiesis and α-granule defect have been described (Hinckley and Di Paola 2014).

Arthrogryposis, Renal Dysfunction, and Cholestasis Syndrome

These patients are characterized by anomalies such as arthrogryposis multiplex congenita, kidney dysfunction,

cholestasis, failure to thrive, and increased bleeding tendency. Usually the patients die during their first year of life. The children present with pale platelets and a pronounced shortage or absence of α-granules (Kim et al. 2010). Aggregation after stimulation with ADP and arachidonic acid is pathological. Arthrogryposis, renal dysfunction, and cholestasis (ARC) syndrome is caused by defects in the VPS33B protein, a Sec1/Munc protein. VPS33B protein is a membrane-associated protein that regulates the SNARE proteins and is involved in biogenesis of granules. This disease is inherited via the autosomal-recessive pathway.

Quebec Platelet Defect

Quebec platelet defect is characterized by the upregulation of urokinase plasminogen activator in platelet α-granules (Kahr et al. 2001), which leads to increased levels of u-plasminogen activator and to exaggerated fibrinolytic activity, that is, the increased depletion of α-granule proteins via plasmin. This defect, which is inherited via the autosomal dominant pathway, is attributable to a duplication in the *PLAU* (urokinase plasminogen activator) gene. Affected patients suffer from severe bleeding that fails to respond adequately to platelet administration; however, they do respond to antifibrinolytics.

X-Chromosomally Inherited Macrothrombocytopenia

Patients with a mutation in GATA1 present with thrombocytopenia, gray platelets, and very reduced or absent α-granules. The hypothesis is that GATA1 is a regulator of several genes lying upstream that are responsible for α-granules biogenesis. As GATA1 is important as a transcription factor for megakaryocytopoesis and erythropoiesis, these patients have anemia as well as thrombocytopenia (Nichols et al. 2000).

Secretion Disorder Affecting δ-Granules

Following platelet activation, the δ-granules release their contents (ADP, ATP, and serotonin) and thus contribute to increased platelet activation. 10–18 % of patients with a congenital platelet functional disorder suffer from a secretion disorder of the δ-granules whose genetic origin is currently unknown. The assumption is that the platelet secretion disorder is caused by various genetic anomalies. δ-Granule secretion defect seems to be inherited either via the autosomal recessive or autosomal dominant pathway. The degree of bleeding tendency varies widely. The diagnosis of this disorder using platelet aggregometry yields highly variable results, which is why it is important to employ flow cytometry analyses to diagnose the secretion disorder (decreased CD63 expression). The platelet concentration of adenin nucleotides and the ADP/ATP quotient are both reduced.

Hermansky-Pudlak Syndrome

Patients with Hermansky-Pudlak syndrome are characterized by tyrosinase-positive oculocutaneous albinism and increased bleeding symptoms which are caused by a δ-granule defect (Gahl et al. 1998). These patients suffer from poor vision and nystagmus. They can also develop inflammatory intestinal diseases and/or interstitial lung fibrosis. Accumulation of ceroid-like material in various tissues has been described and is caused by defect involving the organelles in different cell systems. Platelet δ-granules, melanosomes, and lysosomes are affected. Most patients present prolonged bleeding time (Ivy) and pathological δ-granule secretion. The disease is inherited via the autosomal recessive pathway, triggered by a mutation on one of the ten *HPS* genes. All the HPS proteins are members of the four protein complexes: BLOC-1 (biogenesis of lysosome-related organelle complex-1), BLOC-2, BLOC-3, and adaptor protein complex-3 (AP-3).

HPS type 2 (HPS2) is caused by mutations in the gene encoding the β-subunit of the cytosolic adaptor protein AP-3 (*AP3B1*). The AP-3 complex is important for the formation of vesicles at the early endosomes and is ubiquitously expressed. This complex is responsible to recruit cargo proteins to newly formed vesicles and transport them to late endocytic compartments. The absence of the AP-3 complex leads to missorting of various proteins from specialized intracellular compartments to the general exocytotic pathway. Patients with HPS2 develop an immune deficiency in addition to the other symptoms. Protein missorting leads to several features of the HPS2 phenotype (neutropenia, pigmentation disorder, platelet secretion defect). Patients with HPS2 can be distinguished by platelet flow analysis from the other HPS subtypes. Flow cytometric analysis of the patient's platelets showed already elevated CD63 expression on resting platelets with no further increase after thrombin stimulation. Misrouting of lysosomal proteins such as CD107 or CD63 to the cell membrane has also been found on fibroblasts and CTL clones. So far, one patient with HPS2 who developed hemophagocytic lymphohistiocytosis (HLH) has been described (Enders et al. 2006). In addition, one patient with a HPS10 defect which caused an immune deficiency has been described (Ammann et al. 2016).

Chediak-Higashi Syndrome

Patients with Chediak-Higashi syndrome present with an immune deficiency (lysosomal defect) due to a mutation in the *LYST* (lysosomal traffic regulator) gene, oculocutaneous albinism, neurological anomalies, and mild hemophilia (platelet δ-granule defect). The protein LYST is involved in trafficking processes of lysosomes and δ-granules. The disease is inherited via the autosomal recessive pathway. Patients suffer from recurrent bacterial infections, leukocytopenia, and pathological natural killer (NK) function (Rendu et al. 1983). Thrombocytopenia may be present due to splenomegaly. The blood smear typically reveals peroxidase-positive inclusions in the neutrophils. Most of these patients develop lymphohistiocytosis. The children tend to die before the tenth year of life unless hematopoietic stem cell transplantation (HSCT) has taken place.

Griscelli Syndrome

Patients with Griscelli syndrome (mutation either in the *RAB27A*, *MYO5A*, or *MLPH* gene) present (in addition to the typical hypopigmentation) with frequent bacterial infections, hepatosplenomegaly, neutro- and thrombocytopenia, and immune deficiency. There is no solid evidence thus far of increased bleeding tendency. The disease is inherited via the autosomal recessive pathway. Patients with a RAB27A mutation still express the *RAB27B* gene (which compensates in part for the RAB27A defect), which may be why their bleeding tendency is not very pronounced. In a mouse model (*ashen*) a reduction of δ-granules has been described (Gunay-Aygun et al. 2004; Sandrock and Zieger 2010).

TAR (Thrombocytopenia with Absent Radius) Syndrome

Typically, patients with TAR are missing the bilateral radii, but their thumbs are present. They also show transient thrombocytopenia ($<50 \times 10^9$/L) and may have a reduced number of δ-granules in platelets (Bonsi et al. 2009). Other potential malformations include skeletal anomalies, heart defects, or malformations in the urogenital tract. TAR syndrome is caused by an at least 200 kb-large deletion on chromosome region 1q21.1, and it is inherited either via an autosomal recessive or a dominant pathway. Most of the patients with TAR carry a rare null allele at the *RBM8A* locus (Albers et al. 2012).

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS), an X-chromosomal, recessively inherited disease, is associated with microthrombocytopenia, a severe immune deficiency, eczematous skin disorders, and myeloproliferative disease (immune histiocytosis) (Gulacsy et al. 2011). Patients can display increased bleeding symptoms, and the severity of the bleeding tendency can vary. Typical are thrombocytopenia and a seriously reduced number of platelet δ-granules. This disease is caused by a mutation in the *WAS* gene which codes for WAS protein (WASP). WASP regulates actin filament organization. Hematopoietic stem cell transplantations have successfully performed in these patients.

Secretion Disorder Affecting α- and δ-Granules

Patients with RUNX1 haplodeficiency present with an autosomal dominant thrombocytopenia, abnormal platelet responses, and an increased predisposition to leukemia. The platelet function and biochemical abnormalities reported with RUNX1 mutations comprise decreased aggregation and α- and δ-granule deficiencies (Hinckley and Di Paola 2014; Stockley et al. 2013; Rao 2013).

Paris-Trousseau Syndrome and Jacobsen Syndrome (11q Terminal Deletion Syndrome)

Patients with Paris-Trousseau syndrome or Jacobsen syndrome usually present with mild tendency to bleed and congenital macrothrombocytopenia. α-Granules are present, but α-granule secretion is highly pathological. In addition, Stockley et al. described a few patients with FLI1 defects and reduction in platelet ATP secretion in response to all agonists (Stockley et al. 2013). The cause is a defect in the *FLI1* gene (Hart et al. 2000; Favier et al. 2003). In the case of Jacobsen syndrome, there is a large deletion on chromosome 11, which incorporates the *FLI1* gene. FLI1 is a member of the ETS (E-twenty six) family of transcription factors and is expressed in hematopoietic cells. FLI1 regulates genes expressed in early and late megakaryocytopoiesis. These patients can additionally present a congenital heart defect, facial dysmorphia, and psychomotoric retardation.

Therapy

In case of a bleeding, hemostyptical measures locally are important. Other potential therapies are antifibrinolytics or DDAVP (desmopressin, only in children over 3 years of age) (Hayward et al. 2006). Recombinant FVIIa or platelet concentrate can also be administered. However, after application of platelet concentrates, the patients can develop antibodies against platelets and HLA antigens, respectively. Patients who develop anemia should undergo iron substitution. To achieve normalization of the hematocrit is important as the red blood cells have the ability to force platelets onto the vessel wall so that they adhere to the injured endothelium. These patients had been suffering from very severe, life-threatening bleeding symptoms and therefore, HSCT was performed.

Take Home Message

Patients with thrombocytopenia and/or thrombocytopathia should be further investigated using blood smear, platelet aggregometry (Born), and flow cytometry. It is important to distinguish micro- and macrothrombocytopenia. Analyses of platelet receptors expression and their function can hint to the diagnosis. In addition, P-selectin (CD62) is a marker for α-secretion defect and CD63 is used to identify a δ-granule secretion defect in platelets.

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Defects of Platelet Signal Transduction

Binggang Xiang and Zhenyu Li

Abstract

Blood platelets play an essential role in hemostasis. The primary physiological function of platelets is to form hemostatic thrombi that prevent blood loss and maintain vascular integrity, which requires a robust and precise response of platelets to vascular injury. On the other hand, platelet activation must be tightly controlled. Dysregulated thrombus formation (thrombosis) causes blockage of blood vessels, leading to ischemia. Platelets in normal circulation are in a non-adherent "resting" state. Upon vascular injury, platelets are activated following initial adhesion to adhesive proteins like von Willebrand factor (VWF) and collagen or by soluble platelet agonists like ADP, thrombin, and thromboxane A_2 (TXA_2). These adhesive proteins and soluble agonists induce signal transduction via their respective receptors and receptor-specific signaling pathways. The various receptor-specific platelet activation signaling pathways converge into common signaling events, such as activation of PKC and Ca^{2+} mobilization, which stimulate platelet shape change, granule secretion, and ultimately induce the "inside-out" signaling process leading to activation of the ligand binding function of integrin $\alpha_{IIb}\beta_3$. Ligand binding to integrin $\alpha_{IIb}\beta_3$ mediates platelet adhesion and aggregation and triggers "outside-in" signaling, resulting in platelet spreading, granule secretion, stabilization of platelet adhesion and aggregation, and clot retraction. Defects in platelet signal transduction may lead to bleeding disorder. This chapter briefly introduces normal signal transduction of platelet activation followed by detailed description of inherited defects in the signaling molecules in platelets including receptors and kinases, leading to impaired platelet function and bleeding disorders (Table 1).

Defects of Collagen/GPVI/Integrin α2β1 Signaling

Platelets have two major receptors for collagen, integrin $\alpha_2\beta_1$, which is important for platelet adhesion to collagen, and GPVI, which is required for collagen-induced platelet signaling and activation (Clemetson and Clemetson 2001). GPVI is a member of the immunoglobulin superfamily, which is coupled to IgG Fc receptor γ chain (FcRγ) (Tsuji et al. 1997). Upon cross-linking of GPVI by its ligand, the immunoreceptor tyrosine-based activation motif (ITAM), YxxL/I-X_{6-8}-YXXL/I, within FcRγ cytoplasmic domain, is tyrosine phosphorylated by Src family kinases (SFKs) (mainly Lyn and Fyn) bound to the cytoplasmic domain of GPVI (Ezumi et al. 1998; Quek et al. 2000). ITAM phosphorylation leads to binding and activation of the tyrosine kinase Syk, which phosphorylates downstream targets such as the transmembrane adapter linker for activation of T cells (LAT) and the Src homology (SH) 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76). This induces formation of a signaling complex including LAT, SLP-76,

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Table 1 Defects of platelet signal transduction and bleeding disorder

1. Defects of collagen/GPVI/integrin $\alpha_2\beta_1$ signaling
(a) Defects in GPVI gene
(b) Defects in integrin $\alpha_2\beta_1$
(c) Defects in Bruton's tyrosine kinase (Btk)
2. Defects of VWF/GPIb-IX-V signaling
(a) Defects in GPIb-IX-V complex (Bernard-Soulier syndrome)
3. Defects in integrin signaling
(a) Defects in $\alpha_{IIb}\beta_3$ (Glanzmann thrombasthenia)
(b) Defects in kindlin 3 (LAD-III)
(c) Defects in ADAP
4. Defects in platelet activation and signaling mediated by G protein-coupled receptors (GPCRs)
(a) Defects in GPCRs
Defects in TXA_2 receptor
Defects in ADP receptors $P2Y_{12}$ and P2Y1
Defects in ATP receptor P2X1
(b) Defects in Gq signaling
(c) Defects in Gi signaling
(d) Defects in Gs signaling
(e) Defects in the regulator of G protein signaling (RGS)
5. Defects in common platelet activation signaling and amplification pathways
(a) Defects in TXA_2 generation
cPLA2 deficiency
COX-1 deficiency
TXA_2 synthase deficiency
(b) Defects in PKA
(c) Defects in phospholipase C signaling
Defects in phospholipase C β2
(d) Defects in calcium signaling
Defects in CalDAG-GEFI
(e) Defects in procoagulant activity
Defects in TMEM16F (Scott syndrome)
Defects in STIM1(Stormorken syndrome)
(f) Defects in cytoskeletal organization
Defects in WASP (Wiskott-Aldrich syndrome)
(g) Defects in secretion signaling
Defects in syntaxin-11 (FHLH type 4)

Btk, Gads, and phospholipase Cγ2 (PLCγ2), which further activates PLCγ2 (Watson et al. 2005; Nieswandt and Watson 2003), leading to TXA_2 synthesis, granule secretion, and integrin activation (Ezumi et al. 1998; Quek et al. 2000). Signal transducer and activator of transcription 3 (STAT3) is a transcription factor activated by cytokine-induced intracellular signals. A recent study reported that a novel nontranscriptional activity of STAT3 enhanced collagen-induced signaling in platelets (Zhou et al. 2013). STAT3 serves as a protein scaffold to facilitate the activation of PLCγ2 by Syk. Maintenance of a pool of active SFKs in platelets appears to be important for GPVI-mediated platelet activation and involves CD148, a receptor-like protein tyrosine phosphatase (PTP), which has been reported to dephosphorylate the C-terminal inhibitory tyrosines of SFKs (Senis et al. 2009). The PH domain of PLCγ2 also interacts with the PI3K product phosphatidylinositol 3,4,5-trisphosphate (PI 3,4,5P3), thereby facilitating PLCγ2 recruitment to the plasma membrane and activation (Pasquet et al. 1999; Watanabe et al. 2003; Gilio et al. 2009). Platelet ITAM signaling appears critical for vascular integrity in inflammation (Boulaftali et al. 2013).

Integrin $\alpha_2\beta_1$ plays a critical role in adhesion of platelets to collagen and the stabilization of platelet-collagen interactions. Although megakaryocyte- and platelet-specific β1 conditional knockout mice have normal aggregation to collagen and tail-bleeding times, suggesting $\alpha_2\beta_1$ is not an essential receptor for collagen (Nieswandt et al. 2001), binding of platelets to collagen is reduced when integrin β1 is deleted or platelets are treated with a blocking antibody against $\alpha_2\beta_1$. $\alpha_2\beta_1$ mediates platelet adhesion and spreading on collagen via a pathway involving multiple signaling events, including Src, Syk, SLP-76, PLCγ2, FAK, plasma membrane calcium ATPase, and Ca^{2+} mobilization (Inoue et al. 2003). Under flow conditions, efficient platelet adhesion on collagen requires both integrin $\alpha_2\beta_1$ and GPVI (Sarratt et al. 2005). SLP-76 but not LAT plays an important role in platelet adhesion on collagen under flow.

Defects in GPVI gene have been reported previously in several unrelated patients (Dumont et al. 2009; Hermans et al. 2009; Matus et al. 2013). A patient with an R38C mutation in exon 3 of one allele and an insertion of five nucleotides in exon 4 of the other allele of the gene coding GPVI had a mild bleeding phenotype (Dumont et al. 2009). Platelets from the patient had no response to collagen stimulation. The expression level of GPVI in platelets was dramatically reduced. In contrast to wild-type recombinant GPVI-Fc, the ability of R38C GPVI-Fc to bind to collagen was diminished. Another patient was compound heterozygous for an out-of-frame 16-bp deletion and a missense mutation S175N (Hermans et al. 2009). The patient had a lifelong history of bleeding problem. Platelets from this patient did not respond to collagen, convulxin, or the collagen-related peptide (CRP). Patients lacking integrin $\alpha_2\beta_1$ showed prolonged bleeding (Nieuwenhuis et al. 1985). Bruton's tyrosine kinase (Btk) plays a crucial role in B-cell development. Lack of Btk expression due to mutations in the gene coding Btk in humans leads to the B-cell deficiency X-linked agammaglobulinemia (XLA). Btk is also expressed in platelets and plays roles in collagen- and VWF-induced platelet activation (Liu et al. 2006; Quek et al. 1998). Platelet aggregation and secretion in response to collagen in the XLA patients were significantly diminished (Quek et al. 1998).

Defects of VWF/GPIb-IX-V Signaling

Under high shear rate flow conditions, initial platelet adhesion requires binding of immobilized VWF to its receptor, GPIb-IX-V complex on platelets (Savage et al. 1996; Lopez and Dong 1997; Du 2007). VWF forms a so-called catch-bond or flex-bond with the ligand binding domain of GPIbα (Yago et al. 2008; Kim et al. 2010), allowing transient platelet adhesion under high shear stress. VWF/GPIb-IX-V interaction induces signaling events, leading to integrin activation and integrin-dependent stable platelet adhesion and aggregation (Du 2007). In addition, GPIb-IX-V binds thrombin and sensitizes platelets to low-dose thrombin (Estevez et al. 2016).

There has been evidence that GPIb-IX-V is associated with the ITAM receptors Fcγ receptor IIA (FcγRIIA) (Sullam et al. 1998) or FcRγ (Wu et al. 2001). Genetic deletion of ITAM signaling molecules, such as FcRγ, LAT, SLP-76, and Btk, abolishes the TXA_2- and secretion-dependent second wave of platelet aggregation induced by VWF/botrocetin in washed mouse platelets (Liu et al. 2005, 2006). However, loss of FcRγ and LAT does not appear to affect GPIb-IX-V-dependent integrin activation (Liu et al. 2005). Similarly, Syk is not required for GPIb-IX-V- and integrin-dependent stable platelet adhesion to VWF under shear stress (Yin et al. 2008b). Considering the importance of the ITAM pathway in granule secretion and integrin outside-in signaling, it likely functions as an important signal amplification mechanism in GPIb-IX-V signaling.

The cytoplasmic domain of the GPIbα chain interacts with SFKs and PI3Ks (Wu et al. 2001; Mu et al. 2010), which are both important for transmitting the "early" activation signals from GPIb-IX-V (Yap et al. 2002; Kasirer-Friede et al. 2004; Mu et al. 2010; Liu et al. 2005; Yin et al. 2008a). The SFK Lyn is required for activation of PI3K and its downstream effector Akt, leading to integrin activation (Liu et al. 2005; Yin et al. 2008a, b). Interestingly, VWF/GPIb-IX-V interaction induces elevation of intracellular cGMP levels (Li et al. 2003b; Riba et al. 2006), which sequentially activate the cGMP-dependent protein kinase (PKG) and the mitogen-activated protein kinases (MAPK) p38 and ERK (Li et al. 2001, 2003b, 2006). GPIb-IX-V-dependent activation of the cGMP pathway is downstream of the Lyn-PI3K-Akt pathway (Yin et al. 2008a, b), which is known to activate nitric oxide (NO) synthase (NOS). VWF-induced cGMP elevation may involve both NO-dependent (Li et al. 2003b; Riba et al. 2006) and NO-independent mechanisms (Gambaryan et al. 2008). It is important to note that the role of NO and cGMP in platelet activation is biphasic (Li et al. 2003b). The low concentrations of NO/cGMP synthesized endogenously during platelet activation are stimulatory, whereas high concentrations of NO and cGMP inhibit platelet activation. The biphasic role of the NO/cGMP pathway may serve to stimulate robust hemostatic thrombus formation at sites of vascular injury while preventing overgrowth of the thrombus.

Defects in the GPIb-IX-V complex result in Bernard-Soulier syndrome (BSS). BSS is characterized by prolonged bleeding time, thrombocytopenia, increased megakaryocytes, and enlarged platelets. The large platelets and low platelet count in BSS are due to the absence of GPIbα and the filamin A binding site that links the GPIb-IX-V complex to the platelet membrane skeleton, because the enlarged platelet abnormality and low platelet count have been reversed in GPIbα knockout mice by the expression of a chimeric GPIbα composed of the extracellular human IL-4 receptor and the cytoplasmic tail of GPIbα (Kanaji et al. 2002). Mutants in the genes coding GPIbα, GPIbβ, or GPIX can cause a lack of expression of the GPIb-IX-V complex or abnormalities of GPIb-IX-V function, resulting in BSS.

Defects in Integrin Signaling

Integrin Signaling

Integrin Inside-Out Signaling

Integrins expressed in platelets include $\alpha_{IIb}\beta_3$ (fibrinogen receptor), $\alpha_v\beta_3$ (vitronectin receptor), $\alpha_2\beta_1$ (collagen receptor), $\alpha_5\beta_1$ (fibronectin receptor), and $\alpha_6\beta_1$ (laminin receptor). $\alpha_{IIb}\beta_3$ is the most abundant integrin in platelets. $\alpha_{IIb}\beta_3$ is normally kept in a "resting" or low-affinity state in circulating platelets, but transforms into a high-affinity "activated" state following platelet activation. Activated $\alpha_{IIb}\beta_3$, by binding to its ligands (fibrinogen, VWF, and many matrix proteins containing RGD-like sequences), mediates stable platelet adhesion, aggregation, and thrombus formation. The integrin-proximal intracellular signaling mechanism that induces changes in the extracellular ligand binding domain of integrins from a "low-affinity" state to the "activated" state is referred to as "inside-out" signaling. Inside-out signaling requires the binding of talin and kindlins to the cytoplasmic domain of β3 (Tadokoro et al. 2003; Moser et al. 2008; Ma et al. 2008). Talin and kindlins bind to different sites in the β_3 cytoplasmic domain. Talin binds to the membrane-proximal region and the membrane-proximal NXXY motif of β_3 (Calderwood et al. 1999; Patil et al. 1999; Wegener et al. 2007), but kindlins bind to the sequences around the C-terminal NXXY motif (Moser et al. 2008; Ma et al. 2008). The possible role of integrin transmembrane domain interactions in this process has also been suggested (Li et al. 2003a). Defects in integrin inside-out signaling result in reduced ligand binding function of integrin and deficient platelet aggregation.

CalDAG-GEF1 and its downstream target, Rap1, play an important role in inside-out signaling (Crittenden et al. 2004; Chrzanowska-Wodnicka et al. 2005). CalDAG-GEF1 converts Rap1, a member of Ras family of small GTPases, from the GDP-bound form to the active GTP-bound form, which interacts with the Rap1-GTP-interating adaptor molecule (RIAM). The role of CalDAG-GEF1/Rap1 in integrin inside-out signaling is consistent with the data that RIAM promotes $\alpha_{IIb}\beta_3$-talin interaction and integrin activation (Lafuente et al. 2004). Rap1b has also been shown to be involved in $\alpha_{IIb}\beta_3$ outside-in signaling (Zhang et al. 2011).

Outside-In Signaling

Ligand binding to integrin $\alpha_{IIb}\beta_3$ mediates platelet adhesion and aggregation and also initiates a series of intracellular signaling events ("outside-in" signaling), leading to platelet spreading, granule secretion, stable adhesion, and clot retraction (Shattil and Newman 2004). Defects in integrin outside-in signaling result in reduced platelet spreading, adhesion, and clot retraction. Following ligand binding, integrins undergo "a ligand-induced conformational change" that can be propagated outside-in to the cytoplasmic domain (Leisner et al. 1999). However, although ligand-induced conformational changes of $\alpha_{IIb}\beta_3$ occur with either the multimeric macromolecular ligands such as fibrinogen or monomeric peptide ligands such as RGDS, significant cellular response only occurs with multimeric macromolecular ligands, suggesting that ligand-induced receptor clustering may be important for transmitting outside-in signals. The most proximal signaling event that occurs following integrin ligation is the binding of the G protein subunit $G\alpha_{13}$ to the cytoplasmic domain of β3 (Gong et al. 2010). $G\alpha_{13}$ and talin bind to mutually exclusive but distinct sites within the integrin β_3 cytoplasmic domain in opposing waves. A recent study reported a novel integrin binding protein, VPS33B, a member of the Sec1/Munc18 (SM) family. VPS33B binds directly to the integrin β subunits and is required specifically for $\alpha_{IIb}\beta_3$ outside-in signaling (Xiang et al. 2015).

Defects in Integrin Signaling

Defects in $\alpha_{IIb}\beta_3$

$\alpha_{IIb}\beta_3$ is coded by two genes *ITGA2B* and *ITGB3*. Defects in either α_{IIb} or β_3 lead to a bleeding disorder called Glanzmann thrombasthenia (GT). Platelets from GT patients fail to aggregate in response to all physiological agonists due to quantitative or qualitative defects in the $\alpha_{IIb}\beta_3$ integrin (Nurden et al. 2011; Nurden and Nurden 2015; Sandrock-Lang et al. 2015). Bleeding tendency in GT patients is variable but may be severe.

Defects in Kindlin 3

Leukocyte adhesion deficiency (LAD) is a rare autosomal recessive disorder characterized by immunodeficiency resulting in recurrent infections. LAD is currently divided into three subtypes, LAD-I, LAD-II, and LAD-III, due to deficiency of β2 integrin, GDP-fucose transporter 1, and kindlin-3, respectively. Because kindlin-3 affects multiple integrin function including $\alpha_{IIb}\beta_3$, LAD-III patients have a Glanzmann thrombasthenia-like bleeding tendency (Malinin et al. 2009; Svensson et al. 2009; Jurk et al. 2010; Meller et al. 2012; Crazzolara et al. 2015).

Defects in ADAP

Adhesion and degranulation promoting adapter protein (ADAP) is an adapter protein that plays a role in inside-out activation of the integrin LFA-1 (αLβ2) in T cells. ADAP is also expressed in platelets and promotes integrin $\alpha_{IIb}\beta_3$ activation through interactions with talin and kindlin-3 (Kasirer-Friede et al. 2014). A homozygous nonsense mutation (c.393G>A) in the ADAP coding gene, FYB, is associated with reduced platelet activation by ADP. The patient lacking ADAP had thrombocytopenia and a significant bleeding tendency (Levin et al. 2015).

Defects in Platelet Activation and Signaling Mediated by G Protein-Coupled Receptors (GPCRs)

Platelet Activation and Signaling Mediated by GPCRs

A variety of soluble platelet agonists are released from damaged cells (e.g., ADP), produced during coagulation (e.g., thrombin) and inflammation [e.g., platelet-activating factor (PAF)], and enriched in atherosclerotic plaques [e.g., lysophosphatidic acid (LPA)]. These agonists play critical roles in platelet activation and thrombus formation (Offermanns 2006). Equally important, soluble platelet agonists, TXA_2, ADP, and serotonin are released from stimulated platelets and serve to amplify platelet activation. All these agonists activate platelets via GPCRs, a family of seven-transmembrane domain receptors that transmit signals through heterotrimeric G proteins (Fig. 1).

The heterotrimeric G proteins consist of three subunits, α, β, and γ, which bind to GPCR in an α/β/γ complex. Upon receptor ligation, the α subunit is converted from a GDP-bound form to the active GTP-bound form. Activated Gα subunits dissociate from the receptor and from the β/γ complex and interact with specific downstream targets to transmit GPCR signals. Based on the similarity of the α subunits, G proteins can be divided into four subfamilies:

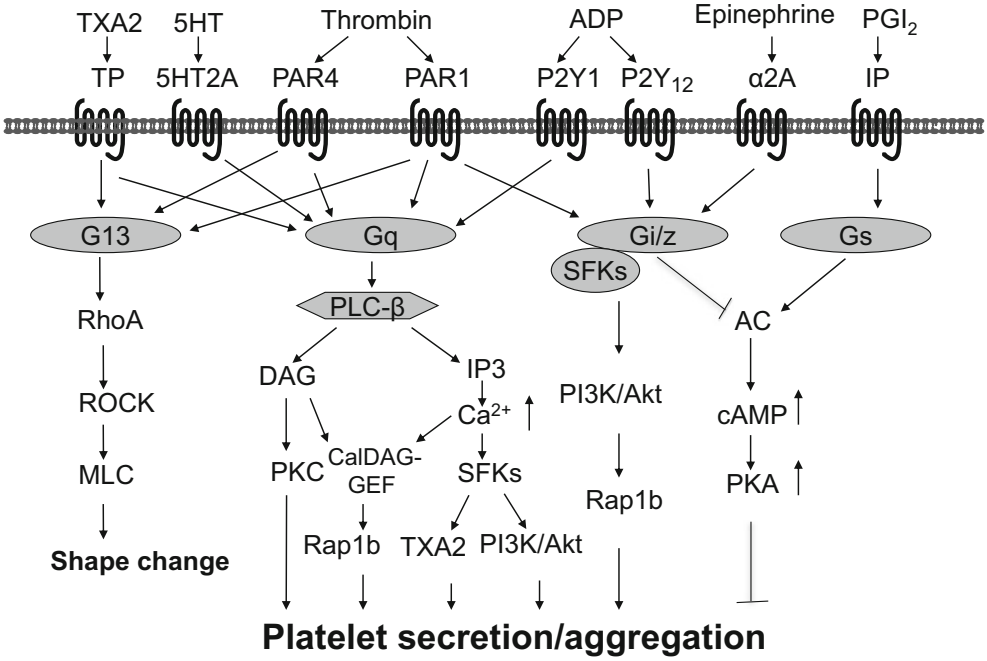

Fig. 1 GPCR signaling in platelets

G_q/G_{11}, G_{12}/G_{13}, $G_i/G_o/G_z$, and G_s, each of which couples to selective receptors and downstream effectors (Fig. 1) (Offermanns 2006). Platelets express G_q, G_{12}/G_{13}, $G_i/_z$, and G_s. G proteins in platelets couple to agonist receptors that stimulate platelet activation, with the exception of G_s. G_s couples to receptors for physiological platelet inhibitors (PGI2 and adenosine) that mediate inhibitory signals by stimulating adenylyl cyclase-dependent cAMP synthesis. Thrombin-induced platelet activation is mediated via a dual system of G protein-coupled protease-activated receptors (PARs): PAR1 and PAR4 in humans (Coughlin 1999) and PAR3 and PAR4 in mice (Kahn et al. 1998). PAR3 appears to sensitize PAR4 to thrombin (Nakanishi-Matsui et al. 2000; Sambrano et al. 2001). PAR1 and PAR4 directly couple to G_q and G12/G13 (Kahn et al. 1998) and probably also G_i (Coughlin 2005). TXA_2 activates platelets via the TXA_2 receptor TP, which couples to G_q and G_{13} (Knezevic et al. 1993; Djellas et al. 1999). ADP induces platelet activation via P2Y1 (G_q-coupled) and $P2Y_{12}$ (G_i-coupled) (Offermanns 2006). Serotonin recognizes the G_q-coupled receptor 5HT2A (Offermanns 2006).

G_q transmits cellular signals mainly through its interaction and stimulation of phospholipase C-β (PLCβ). G_q signaling is important for GPCR-stimulated platelet granule secretion, integrin activation, and consequent platelet aggregation (Offermanns et al. 1997). Deletion of G_q causes defects in platelet secretion and aggregation in response to a variety of agonists including thrombin, ADP, TXA_2 analog U46619, and even collagen (probably due to the dependence of the collagen signaling pathway on TXA_2) (Offermanns et al. 1997). Two alternative signaling pathways, the Ca^{2+}/SFKs/PI3K and PKC, mediate Gq-dependent platelet activation (Xiang et al. 2012).

Cyclic nucleotide cAMP is an important second messenger in platelets and plays a critical role in regulating platelet function. cAMP activates the cAMP-dependent protein kinase (PKA) to inhibit platelet activation. Intracellular cAMP levels in platelets are regulated by G_i and G_s. While platelet agonists such as ADP activate G_i to decrease cAMP levels, prostaglandin E1 (PGE1) and PGI2 inhibit platelet function through elevating intra-platelet cAMP levels via activating their G_s-coupled receptors. Optimal platelet activation also requires G_i signaling, especially in response to weak or low-dose platelet agonists. The G_i-coupled ADP receptor, $P2Y_{12}$ (Hollopeter et al. 2001; Foster et al. 2001), is required for ADP-induced platelet activation and also promotes platelet activation induced by TXA_2 and low-dose thrombin. $P2Y_{12}$ couples to G_{i2} (Ohlmann et al. 1995; Jantzen et al. 2001) and the epinephrine receptor in platelets couples to G_z, another G_i subtype (Yang et al. 2000). The importance of G_i-coupled receptors in promoting platelet activation is consistent with the inhibitory effect of G_i on cAMP synthesis. In addition to the inhibition of cAMP synthesis, G_i also plays a role in the activation of the PI3K/Akt signaling pathway and the small GTPase Rap1b (Fig. 1) (Woulfe et al. 2002; Lova et al. 2003).

G_{13} also plays a role in platelet activation. G_{13} knockout platelets show partial reduction in platelet secretion and unstable platelet aggregation induced by low-dose thrombin and the TXA_2 analog U46619 (Moers et al. 2003). Shape change induced by these agonists is also abolished in G13

knockout platelets. GTP-bound G_{13} interacts with and activates guanine nucleotide exchange factors (GEF) for the small G protein RhoA, such as p115RhoGEF, which subsequently converts RhoA into the active GTP-bound form (Kozasa et al. 1998). RhoA activates Rho kinase, which phosphorylates and inhibits myosin light chain (MLC) phosphatase (Klages et al. 1999), consequently, enhancing MLC phosphorylation and MLC-dependent contraction. Thus, G_{13} stimulates platelet shape change and granule secretion (Klages et al. 1999). However, the relatively mild in vitro aggregation defect in G_{13} knockout platelets is contrasted with the dramatic defects in hemostasis and thrombosis in vivo, suggesting an additional role of G_{13} in platelet-dependent thrombus formation (Moers et al. 2003). Indeed, G_{13} also binds to the cytoplasmic domain of integrin β3 and plays a critical role in integrin outside-in signaling (Gong et al. 2010).

Defects in GPCR Signaling

Defects in GPCRs

Defects in TXA_2 Receptor

Mutations in GPCRs have been identified in patients with bleeding. A single amino acid substitution R60L in the first cytoplasmic loop of the TXA_2 receptor was found in two unrelated families. Although the ligand binding affinity was not affected, R60L substitution in the TXA_2 receptor resulted in impaired PLC activation in response to a TXA_2 analog in recombinant Chinese hamster ovary cells (CHO) (Hirata et al. 1994). Platelets from heterozygous R60L mutation showed impaired platelet response to TXA_2 as well as to ADP or epinephrine, although not as severe as that in the homozygote. D304N mutation in the TXA_2 receptor gene has been reported to cause a mild mucocutaneous bleeding in another patient (Mumford et al. 2010). U46619 did not increase cytosolic free Ca^{2+} concentration in CHO cells expressing the variant D304N TXA_2 receptor (Mumford et al. 2010). D304 is located within a conserved NPXXY motif in transmembrane domain 7 of the TXA_2 receptor. D304N substitution leads to impaired ligand binding, suggesting a role of this domain in the ligand binding function of the receptor. A W29C in the TXA_2 receptor results in reduced ligand binding function of the receptor, and U46619 was less potent at inducing Ca^{2+} mobilization in HEK293 cells expressing the W29C receptor than in WT controls (Mumford et al. 2013). A heterozygous c.167dupG mutation in the TXA_2 receptor leading to frameshift was identified in a family. Patients carrying this mutant had bleeding tendency and impaired platelet aggregation, secretion, and integrin activation in response to U46619 (Kamae et al. 2011). A heterozygous N42S mutation in the TXA_2 receptor resulted in reduced surface expression of the receptor and platelet dysfunction (Nisar et al. 2014). The patient had significant bleeding phenotype.

Defects in ADP Receptors $P2Y_{12}$ and $P2Y_1$

Mutations in the ADP receptor $P2Y_{12}$ can also cause bleeding disorder. Patients with homozygous mutations in $P2Y_{12}$ gene that produce a frameshift and premature truncation of the protein had a bleeding tendency (Cattaneo 2005). Another patient with an allele of a frameshift mutation in the $P2Y_{12}$ gene and a normal allele also had a bleeding tendency (Cattaneo 2005). A patient with a compound heterozygote in the $P2Y_{12}$ gene with one allele containing a G to A transition resulting in R256Q substitution and the other allele containing a C to T transition resulting in an R265W substitution had reduced and reversible aggregation in response to ADP (Cattaneo 2005). ADP failed to inhibit adenylyl cyclase activity stimulated by PGE1. A patient carrying a heterozygous mutation of P341A in the $P2Y_{12}$ gene has a mild bleeding disorder (Cunningham et al. 2013). It is found that P341 is important for $P2Y_{12}$ recycling. Another patient with a homozygous H187Q mutation in the $P2Y_{12}$ had bleeding diathesis (Lecchi et al. 2015). ADP-induced platelet aggregation was markedly reduced and rapidly reversible in this case. A patient with a homozygous R122C mutation in $P2Y_{12}$ plus an intronic polymorphism in the thrombin receptor PAR1 coding gene has been reported recently (Patel et al. 2014). Platelet response to ADP or thrombin was reduced. The patient had a chronic bleeding disorder. Mutations in thrombin receptors can also cause bleeding disorder. A mutation of Y157C in the thrombin receptor PAR4 resulted in reduced thrombin-induced platelet responses (Nurden and Nurden 2015). Platelet aggregation and secretion in response to epinephrine was impaired in family members with deficiency of the epinephrine receptor alpha 2-adrenergic receptor (Rao et al. 1988). The patients had slightly prolonged bleeding times. A patient with a history of bleeding following surgery was associated with reduced platelet $P2Y_1$ mRNA. No mutation was detected in the gene coding $P2Y_1$. ADP-induced platelet aggregation was reduced (Cattaneo 2011).

Defects in ATP Receptor P2X1

P2X1 belongs to the family of purinoceptors for ATP. A patient carrying a mutant in P2X1 that lacks one leucine within a stretch of four leucine residues in its second transmembrane domain (amino acids 351–354) showed a selective impairment of the ADP-induced platelet aggregations (Oury et al. 2000). Voltage-clamped HEK293 cells expressing mutated P2X1 channels failed to develop an ATP- or ADP-induced current. When co-expressed with the wild-type receptor in *Xenopus* oocytes, the mutated protein exhibited a dominant negative effect on the normal

ATP- or ADP-induced P2X1 channel activity. The patient had severe bleeding disorder.

Defects in Gq Signaling

Gabbeta et al. reported a patient with deficiency of Gq in platelets (Gabbeta et al. 1997). Platelet aggregation and secretion in response to multiple agonists were defective in this patient. Agonist-induced release of arachidonic acid from phospholipids and calcium mobilization are impaired upon platelet activation. The amount of dense granule contents was normal. Although no mutations are detected in the G_q cDNA, mRNA and protein levels are decreased more than 50 % compared to normal subjects, suggesting that impaired platelet activation and bleeding phenotype of this patient are due to the quantitative abnormality of G_q.

Defects in Gi Signaling

Patel et al. reported that a patient with a chronic bleeding disorder was due to deficiency of G_{i1} in platelets (Patel et al. 2003). Although no mutation was identified in the G_{i1} gene, the expression level of G_{i1} in platelets from the patient was reduced to 25 %, compared to that in normal platelets. The expression levels of G_{i2}, G_{i3}, G_z, and G_q in the patient platelets were normal. Platelet aggregation stimulated by weak agonists such as ADP and adrenaline was severely impaired. Forskolin-stimulated rise in cyclic AMP (cAMP) was impaired. These data suggest that impaired platelet activation and bleeding phenotype of this patient are due to the quantitative abnormality of G_{i1}.

Defects in Gs Signaling

Hyperfunction of G_s has been described in several patients with bleeding disorder. The α subunit of the G protein Gs is encoded by a complex imprinted gene cluster called GNAS1, which is located in the chromosome 20q13 region. This gene has four alternative first exons including an extra-large (XL)-exon1 that correspond to different promoters (Hayward et al. 1998). The four different exons splice to the common exons 2–13 of the classical $G_{s\alpha}$ gene. In three patients, a 36 bp insertion and two base pair substitutions flanking this insertion were found in the paternally inherited XL-GNAS1 exon 1. Platelets from those patients display G_s hyperfunction as evidenced by enhanced cAMP generation upon stimulation of G_s-coupled receptors. All the patients had enhanced trauma-related bleeding tendency (Freson et al. 2001).

Defects in the Regulator of G Protein Signaling

Regulator of G protein signaling 2 (RGS2) negatively regulates Gs signaling by inhibiting the activation of adenylyl cyclase (Roy et al. 2006; Sinnarajah et al. 2001). Noe et al. reported a G23D heterozygous RGS2 mutation in three related patients, leading to Gs hypofunction in their platelets (Noe et al. 2010). cAMP production after stimulation of the G_s-coupled receptors was reduced. Platelet shape change was reduced in the patients. However, platelet aggregation was normal and no bleeding phenotype was observed in the patients.

Defects in Common Platelet Activation Signaling and Amplification Pathways

The initial signaling upon stimulation of various platelet receptors ultimately converges into common intracellular signaling events. In particular, almost all agonists induce activation of PLC (Varga-Szabo et al. 2009). PLC catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP2) to release inositol trisphosphate (IP3) and diacylglycerol (DAG), which activate calcium mobilization and PKC, respectively. All platelet agonists can induce platelet secretion and synthesis of TXA_2, two major amplification mechanism of platelet activation.

The critical role of cytosolic calcium elevation in platelet activation and function has been known for many years. Agonist-induced calcium elevation is mainly induced by IP3 receptor-mediated release of calcium from intracellular stores and store-operated calcium entry from outside of platelets (Hassock et al. 2002; Bergmeier and Stefanini 2009). A role for the store-independent calcium entry has also been shown (Hassock et al. 2002). Certain canonical transient potential channels (TRPC) and calcium release-activated channel (CRAC) (Orai1) have been shown to mediate calcium entry (Hassock et al. 2002; Bergmeier and Stefanini 2009). Elevation of calcium levels activates multiple signaling events and molecules including actin-myosin interaction, protein kinase C (PKC), calmodulin, nitric oxide synthases (NOS), and calcium-dependent proteases. Calcium elevation also positively regulates SFKs and the PI3K/Akt signaling pathway (Xiang et al. 2010, 2012).

Platelets express several members of the PKC family, including the classical (or conventional) PKC, α, β, and γ (calcium and DAG dependent); the novel PKC, δ, θ, and η (DAG dependent, calcium independent); and an atypical PKC ζ (calcium and DAG independent) (Murugappan et al. 2004; Konopatskaya et al. 2009; Nagy et al. 2009; Chari et al. 2009; Pula et al. 2006). Classical PKCs, particularly PKC α, play a critical and general role in platelet granule secretion and secretion-dependent aggregation. PKC α has also been shown to regulate Rap1 and integrin signaling in a reconstituted CHO cell model (Han et al. 2006). PKC δ and θ promote dense granule secretion in response to thrombin receptor agonists (Murugappan et al. 2004; Chari et al. 2009; Nagy et al. 2009). Pleckstrin is a major PKC substrate and may possibly be involved in cytoskeleton regulation

(Lian et al. 2009). Although no bleeding disorder has been reported to be due to defects in the genes coding PKCs, a patient with thrombocytopenia and impaired receptor-mediated aggregation was reported to be caused by a defect in a transcription factor called core-binding factor A2 (CBFA2) with reduced PKCθ mRNA levels in platelets and impaired agonist-induced pleckstrin phosphorylation (Sun et al. 2004).

A common platelet response to all agonists is the secretion of granule contents, which further amplifies platelet activation (Reed et al. 2000; Ren et al. 2008). Besides its role in hemostasis, granule secretion plays important roles in inflammation, atherosclerosis, host defense, wound healing, angiogenesis, and malignancy (Blair and Flaumenhaft 2009). Platelet secretion is mediated by soluble NSF attachment protein receptor (SNARE) proteins from granule and plasma membranes. Platelets contain three major types of granules, α-granules, dense granules, and lysosomes (Ren et al. 2008). The SNAREs form transmembrane complexes that mediate membrane fusion and granule cargo release. It has been known that both VAMP-8 (v-SNARE) and SNAP-23 (a t-SNARE class) are important for platelet secretion (Ren et al. 2008). The interaction between SNARE proteins is regulated by their phosphorylation and involves small GTPases such as Rab27.

Defects in TXA_2 Generation

The thromboxane A_2 (TXA_2) is one of the major contributors to the amplification of the initial platelet activation process. The TXA_2 pathway has been a major target in the treatment of cardiovascular disease. The COX1 inhibitor, aspirin, has been widely used as an antithrombotic drug. On the other hand, defects in TXA_2 synthesis result in bleeding disorder. TXA_2 is synthesized from phospholipids through a series of enzyme-dependent metabolic reaction. It has been reported that defects in cPLA2, COX1, or thromboxane synthase lead to a lack of TXA_2, low platelet activation, and bleeding disorder.

cPLA2 Deficiency

Cytosolic phospholipase A2 (cPLA2) hydrolyzes cellular membrane phospholipids to release arachidonic acid, which is the substrate for the synthesis of eicosanoids, including prostaglandins and leukotrienes. A patient with defects in the gene coding cPLA2 had bleeding disorder (Adler et al. 2008). This patient had two heterozygous single base pair mutations and a known SNP in the coding regions of the patient's cPLA2 gene (S111P+R485H; K651R). The expression of cPLA2 and the total PLA2 activity in platelets were diminished. The urinary metabolites of prostacyclin, prostaglandin E2, prostaglandin D2, and thromboxane A_2 were also reduced. Platelet aggregation and secretion induced by ADP or collagen were diminished in this patient but were normal in response to arachidonic acid. The patient had repeated gastrointestinal bleeding.

COX-1 Deficiency

Defects in the gene coding cyclooxygenase 1 (COX-1) (also known as prostaglandin H synthase 1, PGHS-1) can also lead to bleeding disorder. It has been reported that three female patients had a mild bleeding disorder due to defective COX1 activity (Matijevic-Aleksic et al. 1996). Platelet aggregation in response to ADP, collagen, and epinephrine was impaired. Platelet aggregation induced by arachidonic acid was defective, whereas PGH2-induced aggregation was normal. Platelet TXA_2 production in response to arachidonic acid was reduced, whereas they were normal in response to exogenous PGH2. In two of the three patients, the COX-1 protein was undetectable. The amount of the TXA_2 synthase in platelets was normal in all three patients.

TXA_2 Synthase Deficiency

Defryn et al. reported a bleeding disorder due to thromboxane synthase deficiency (Defreyn et al. 1981). Three family members from three successive generations had a moderate bleeding tendency and a functional platelet defect. All three patients had absent aggregation in response to arachidonic acid. ADP-induced platelet aggregation was reversible. They had a prolonged bleeding time. Serum thromboxane B_2 concentrations were significantly reduced.

Defects in PKA

cAMP-dependent protein kinase (PKA) is an important modulator of platelet function. Many substrates of PKA have been identified in platelets, including Rap1B, GPIbβ, VASP, and filamin A. Manchev et al. recently reported a homozygous mutation I74M in PKA leads to macrothrombocytopenia associated with a thrombocytopathy (Manchev et al. 2014). The patients had a bleeding tendency. Platelet activation as evidenced by P-selectin expression and Ca^{2+} mobilization upon stimulation with the PAR4-activating peptide or thrombin was impaired in these homozygous patients. I74IM substitution in PKA was associated with a marked defect in proplatelet formation and a low level in filamin A in megakaryocytes. Overexpression of wild-type PKA in the patient megakaryocytes in vitro rescued the defect in proplatelet formation.

Defects in Phospholipase C Signaling

Activation of phospholipase C is a key signaling event downstream of Gq. Lee et al. reported a patient with a mild inherited bleeding disorder and abnormal platelet activation due to deficiency of phospholipase C β2 isoform (Lee et al. 1996; Mao et al. 2002). Although no mutation was identified in the gene coding phospholipase C β2, the expression level of phospholipase C β2 decreased by two-third, compared to normal subjects. Generation of inositol 1,4,5-trisphosphate, mobilization of intracellular Ca^{2+}, and phosphorylation of pleckstrin in response to several G protein-mediated agonists were reduced in the patient. Agonist-induced platelet aggregation and secretion were defective.

Defects in Calcium Signaling

CalDAG-GEFI has been shown to mediate several important Ca^{2+} responses, including Rap1 activation, ERK activation, TXA_2 synthesis, and granule secretion (Bergmeier and Stefanini 2009). Importantly, a patient carrying a homozygous G248W mutation in CalDAG-GEFI showed severe bleeding phenotype (Canault et al. 2014). Platelets from the patient presented a reduced ability to activate Rap1 and impaired platelet aggregation in response to agonists. Expression of CalDAG-GEFI mutant in HEK293T cells abolished Rap1 activation. Thrombus formation under flow and platelet spreading on immobilized fibrinogen were defective in the patient. Platelets from the patient exhibited a reduced number of filopodia and failed to form lamellipodia on fibrinogen. Platelet adhesion under flow and spreading on fibrinogen were also impaired in the heterozygotes who had normal platelet aggregation and did not suffer from bleeding. Three other unrelated families with mutations in the CalDAG-GEFI coding gene RASGRP2 have since been identified by exome sequencing, including patients carrying a homozygous RASGRP2 c.1490delT giving a frameshift and a stop codon (p.Phe497 fs*22) (Nurden and Nurden 2015). These patients had severe bleeding phenotype and their platelets failed to aggregate.

Defects in Procoagulant Activity

Platelet phosphatidylserine (PS) exposure is critical to trigger coagulation cascade. Agonist-induced platelet PS exposure involves multiple signaling pathways (Lhermusier et al. 2011; Delaney et al. 2014) and requires a transmembrane domain protein, the calcium-dependent lipid scramblase TMEM16F (Suzuki et al. 2010; Yang et al. 2012; Fujii et al. 2015; Brooks et al. 2015). Deficiency of TMEM16F in patients leads to a rare bleeding disorder Scott syndrome, which was first described in 1979 by Weiss et al. (Weiss et al. 1979; Rosing et al. 1985). Patients with Scott syndrome have normal platelet count and structure and normal platelet secretion, aggregation, metabolism, granule content, and adhesion (Satta et al. 1997; Zwaal et al. 2004). However, platelets from patients with Scott syndrome fail to promote fibrin formation in the presence of coagulation factors. In a Scott syndrome patient, a G-to-T homozygous mutation at the splice-acceptor site in intron 12 of the TMEM16F gene was identified, which causes exon 13 skipping, frameshift, and premature termination of translation (Suzuki et al. 2010). Two different mutations were identified in another Scott syndrome patient: a G-to-A mutation at the first nucleotide of intron 6, disrupting the donor splice site consensus sequence of intron 6, and a single-nucleotide insertion in exon 11 (c.1219insT, cDNA numbering from the ATG), resulting in a frameshift and premature termination of translation at codon 411 (Castoldi et al. 2011).

In contrast to Scott syndrome, patients with Stormorken syndrome have spontaneous PS exposure on platelets. Ca^{2+} release-activated Ca^{2+} channels, which are composed of Orai1 proteins located in the plasma membrane, mediate a specific form of Ca^{2+} influx called store-operated Ca^{2+} entry that contributes to the function of many cell types. Orai1 channels are activated by stromal interaction molecule (STIM) 1 and 2 located in the endoplasmic reticulum (Lacruz and Feske 2015). Gain-of-function gene mutations in ORAI1 and STIM1 lead to Stormorken syndrome. Stormorken syndrome is a rare autosomal-dominant disease with mild bleeding tendency. A heterozygous missense mutation in STIM1 exon 7 (R304W) was found in several Stormorken syndrome patients (Morin et al. 2014; Misceo et al. 2014; Nesin et al. 2014). Platelets from the Stormorken syndrome patients had high basal P-selectin expression and high exposure of phospholipids on the outer surface of the plasma membrane. Store-operated Ca^{2+} entry was markedly attenuated in platelets though resting Ca^{2+} levels were elevated. Agonist-induced platelet aggregation and integrin activation as evident by PAC-1 binding were defective in the Stormorken syndrome patients.

Defects in Cytoskeletal Organization

Wiskott-Aldrich syndrome (WAS), an X-linked immunodeficiency disorder, is caused by mutations in the *WAS* gene coding the WAS protein (WASP) (Derry et al. 1994). WASP is a key regulator of actin polymerization in hematopoietic cells (Thrasher 2002). Wiskott-Aldrich syndrome is characterized by recurrent infections, thrombocytopenia, eczema, autoimmunity, and an increased risk of lymphoma (Notarangelo et al. 2008; Puck and Candotti 2006). Patients with Wiskott-Aldrich syndrome are thrombocytopenic and platelet size is smaller than normal. Severe Wiskott-Aldrich syndrome leads to early death because of infections,

hemorrhage, or malignancy. Defects in myosin heavy chain, filamin, tubulin beta 1, or actin 1 genes/proteins can also lead to defective cytoskeletal organization (Freson et al. 2014).

Defects in Secretion Signaling

Except for SNAP proteins, platelets also express another t-SNARE class, syntaxins, including syntaxin-2, 4, 7, and 11. Syntaxin-11 appears to be the most abundant syntaxin in both human and murine platelets. Syntaxin-11 can form SNARE complexes with both VAMP-8 and SNAP-23. Abnormalities in syntaxin-11 are responsible for familial hemophagocytic lymphohistiocytosis (FHLH) type 4, a rare primary immunodeficiency (zur Stadt et al. 2005). A homozygous D58R mutation in syntaxin-11 had a robust defect in agonist-induced secretion. The patient had normal platelet morphology, activation, and cargo levels (Ye et al. 2012). Platelet secretion was normal in the syntaxin-2 and syntaxin-4 single- or double-knockout mice (Ye et al. 2012). These results indicate that syntaxin-11, but not syntaxin-2 or syntaxin-4, is required for platelet exocytosis.

Take Home Message

- Activation of integrin $\alpha_{IIb}\beta_3$ is essential for hemostasis. Defects in either integrin $\alpha_{IIb}\beta_3$ function or proteins that regulate $\alpha_{IIb}\beta_3$ activation (such as kindling 3 or ADAP) result in severe bleeding disorder.
- Defects in collagen receptor signaling (GPVI and integrin $\alpha_2\beta_1$) or in VWF receptor GPIb-IX-V signaling result in bleeding disorder.
- Defects in GPCRs, the TXA2 receptor, ADP receptors $P2Y_1$ or $P2Y_{12}$, ATP receptor $P2X_1$ or defects in the downstream signaling events of GPCRs, including Gq, Gi, Gs, and the regulators of G protein signaling, result in bleeding disorder.
- Defects in common platelet activation signaling and amplification pathways, such as TXA_2 generation, calcium signaling, phospholipase C signaling, platelet procoagulant activity, cytoskeletal organization, or secretion signaling, result in bleeding disorder.

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Alterations of the Platelet Procoagulant or Fibrinolytic Functions

Bernard Payrastre, Marie-Christine Alessi, and Pierre Sié

Abstract

Discovered in the 1980s, the procoagulant function of platelets is due to their inherent ability to provide, following activation, a critical platform at their plasma membrane surface for the activation of blood clotting enzyme complexes to generate thrombin. Upon platelet activation, the asymmetric orientation of membrane phospholipids rapidly collapses resulting in a calcium-dependent exposure of the anionic phospholipid, phosphatidylserine (PS), at the outer platelet surface. Binding of blood clotting enzyme complexes to this procoagulant membrane surface allows a dramatic increase in the rate of conversion of zymogens to active serine proteases and in turn the production of a burst of thrombin leading to fibrin clot formation and to further platelet activation. The energy-independent, calcium-dependent, platelet scramblase activity, which governs the bidirectional exchange of phospholipids between the two leaflets of the bilayer, is essential for PS exposure during platelet activation. The platelet scramblase protein has remained elusive for years until a significant advance was recently made with the identification of TMEM16F, a membrane protein essential for calcium-dependent PS exposure with loss-of-function mutations in Scott syndrome, a bleeding disorder due to an impaired platelet procoagulant activity.

Platelets also have the capacity to interfere with the fibrinolytic system in several ways, and so doing they can modulate the outcome of thrombolytic therapy by delaying reperfusion and mediating reocclusion but also by promoting bleeding complications. Remarkably, a rare autosomal dominant bleeding disorder, the Quebec platelet disorder, is a unique gain-of-function defect in fibrinolysis.

The most clinically relevant aspects of these two platelet functions will be discussed in this chapter.

Platelet Procoagulant Functions

Disruption of Plasma Membrane Phospholipid Asymmetry Following Platelet Activation

In eukaryotic cells, phospholipids are asymmetrically distributed across the bilayer of the plasma membranes. As a general rule, the amine-containing phospholipids, together with phosphoinositides, are enriched on the cytoplasmic surface of the plasma membrane (inner leaflet), while the choline-containing phospholipids and sphingomyelin are

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enriched on the outer leaflet. Maintenance of transbilayer lipid asymmetry, due to specific protein activity, is essential for normal membrane function. Transbilayer phospholipid exchanges via ATP-requiring translocase activities (flippases and floppases) control unidirectional phospholipid transports against a concentration gradient between the two leaflets of the plasma membrane (Daleke 2003; Zwaal et al. 2005). Moreover, an energy-independent, calcium-dependent, scramblase activity governs the bidirectional exchange of phospholipids between the two leaflets of the bilayer (Kodigepalli et al. 2014). This scramblase activity is critical for the activation of the procoagulant function of blood platelets via the so-called platelet factor-3 (Lhermusier et al. 2011). In 1977, Zwaal et al. (1977) noticed that resting intact platelets offer an inert surface to the plasma coagulation system, owing to the virtual absence, on the cell exterior, of the most procoagulant lipid phosphatidylserine (PS). Conversely, they observed that activated platelets disrupt membrane phospholipid asymmetry to allow PS translocation through the plasma membrane, providing a catalytic lipid surface for interacting coagulation factors. Using both purified coagulation factors and external phospholipases, Bevers et al. (1982, 1983) investigated the mechanisms by which platelets activated with thrombin *plus* collagen are able to expose, on their surface, the procoagulant phospholipid PS. This active process occurred in the absence of cell lysis and corresponded to a transfer of the aminophospholipids, PS, and phosphatidylethanolamine (PE), from the inner to the outer leaflet of the plasma membrane. It was also shown that PS exposure required a large increase in cytoplasmic free calcium concentration, as achieved by calcium ionophores or, more physiologically, by the combination of thrombin *plus* collagen (Bevers and Williamson 2010). Upon cytosolic Ca^{2+} increase, the ATP-dependent flippase activity, able to transfer aminophospholipids, mainly PS, from the outer to the inner leaflet is inhibited. However, this is not sufficient to provide a rapid PS exposure, and the activation of phospholipid scrambling is mandatory. This set of data is at the basis of the biochemical characterization of the platelet scramblase activity (Fig. 1). Importantly, this Ca^{2+}-dependent scramblase activity turned out to be deficient in Scott syndrome patients, a rare inherited bleeding disorder with deficiency in platelet procoagulant activity (Rosing et al. 1985a). This observation strongly stimulated the search of the gene encoding the hypothetical scramblase, but, despite considerable efforts, the platelet scramblase remained elusive for years. A major advance has been made recently with the identification of TMEM16F, also called anoctamin 6 (ANO6), a membrane protein essential for Ca^{2+}-dependent PS exposure that carries loss-of-function mutations in Scott syndrome (Suzuki et al. 2010).

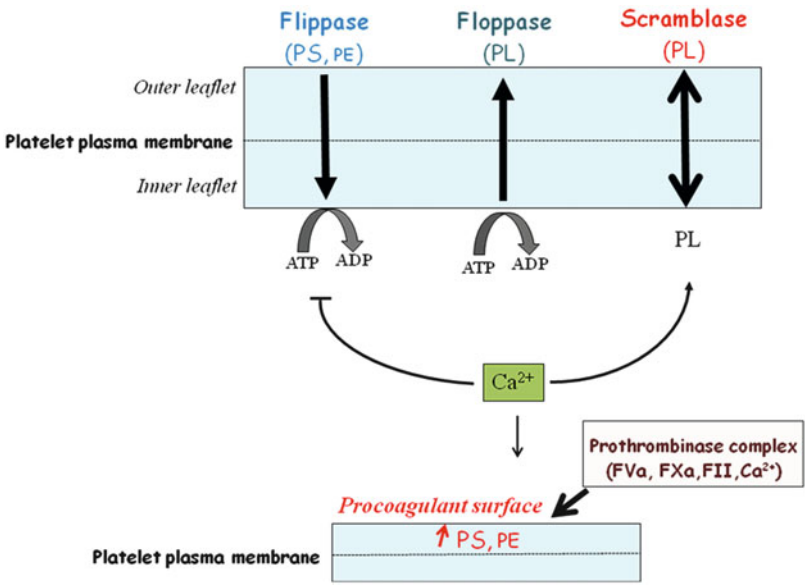

Fig. 1 Phospholipid transport proteins in the platelet membrane and formation of a procoagulant surface. In unstimulated platelets, phospholipid transverse asymmetry is maintained through the action of the two ATP-dependent translocases (flippase and floppase). The external membrane surface consisting almost exclusively of sphingolipids and phosphatidylcholine exerts no procoagulant activity. In contrast, PS, PE, and phosphoinositides are confined to the inner leaflet of the membrane. In the presence of μM Ca^{2+} concentrations (for instance, following thrombin + collagen stimulation), the scramblase catalyzes the bidirectional transfer of phospholipids, while inhibition of flippase contributes to PS exposure on the external surface. This leads to the appearance of a procoagulant surface made of aminophospholipids, mainly PS and PE on the external surface of the plasma membrane. This procoagulant lipid surface interacts with and activates the prothrombinase complex and also the tenase complex (not shown)

PS Exposure as a Critical Event for the Procoagulant Activity of Platelets

As proposed in the 1980s, activated platelets have the capacity to provide at their plasma membrane surface a platform for the activation of blood clotting enzyme complexes to generate thrombin (Tracy et al. 1985; Monroe et al. 2002; Heemskerk et al. 2002; Weiss 2009). Thrombin is a key protease as it cleaves fibrinogen to produce the insoluble fibrin clot and also potently activates platelets, proving an amplification loop of the reaction. Thus, the ability of aminophospholipids, chiefly PS but also PE, to interact with clotting factors at the surface of activated platelets and microparticles amplifies and focuses the clotting reaction at the site of vascular injury, making PS exposure by platelets a critical step in hemostasis.

Following potent activation by soluble agonists, by molecules of the subendothelial matrix, or, more efficiently, by a combination of collagen *plus* thrombin, platelets lose their plasma membrane phospholipid asymmetry leading to PS exposure. Concomitantly, they produce platelet-derived microparticles that also expose PS and PE on their surface (Bevers et al. 1983; Morel et al. 2006). At a physiological pH, the surface exposure of PS provides a negatively charged membrane platform appropriate for the interaction of the nonenzymatic plasma protein factor Va and two other components of the so-called prothrombinase complex, the serine protease factor Xa and the proenzyme factor II (prothrombin). It is thought that the negatively charged membrane binds factor Va in the presence of calcium ions, inducing a conformational change generating a high affinity binding site for factor Xa (Rosing et al. 1980; Bevers et al. 1985). On the other hand, factor Xa and factor II, in the presence of calcium, bind to negatively charged phospholipids by their gamma-carboxylated tail, a product of posttranslational modification dependent on vitamin K in hepatocytes. Assembly of the prothrombinase complex leads to a local propagation of blood coagulation with a rapid and efficient conversion of prothrombin (factor II) to thrombin (factor IIa) through cleavage of two peptide bonds (Husten et al. 1987; Krishnaswamy et al. 1987; Nesheim and Mann 1983; Boskovic et al. 2001). Activated platelets express specific and saturable binding sites for both factors Va and Xa (Rosing et al. 1985b; Bouchard et al. 1997). Compared to factor Xa alone, addition of factor Va into the prothrombinase complex increases the rate of prothrombin cleavage by 5–10 orders of magnitude (Rosing et al. 1985b; Nesheim et al. 1979). Besides providing the membrane surface for assembly of prothrombinase, activated platelets secrete a pool of factor Va which is stored in α-granules of platelets. They also shed microparticles that support prothrombinase complex formation at the vicinity of activated platelets (Sims et al. 1988, 1989; Freyssinet and Toti 2010).

Besides the prothrombinase complex, the tenase complex (VIIIa/IXa), also called "factor Xa-generating complex," interacts with the aminophospholipid surface in a Ca^{2+}-dependent manner which enhances enzymatic activity (Tavoosi et al. 2011; Lentz 2003; Falls et al. 2000). In addition to PS, the role of PE in the assembly and activity of the tenase complex has been documented (Falls et al. 2000). On this phospholipid procoagulant surface, factor IXa and its nonenzymatic cofactor, factor VIIIa, form a complex due to multiple inter-protein contacts (Autin et al. 2005) that converts factor X into factor Xa. Recent data suggest that the interaction with aminophospholipids is not exclusive as factor VIIIa can bind to fibrin on the platelet surface (Gilbert et al. 2015).

Interestingly, aminophospholipids with particular fatty acyl chain composition have been shown to be preferentially externalized suggesting that specific molecular species of PS and PE may preferentially modulate coagulation efficiency (Clark et al. 2013). PE are thought to enhance the rate of interaction of the γ-carboxylated glutamic acids (Gla domains) of vitamin K-dependent coagulation factors with the head group of PS. The fatty acid chain length appears to regulate the ability of PE to favor coagulation, with platelet-specific PE species showing optimum activity.

Regulation of PS Exposure in Platelets

Scrambling activity is not energy-dependent but requires a sustained cytosolic Ca^{2+} increase which acts as a switch for the scrambling machinery (Bevers and Williamson 2010; Williamson et al. 1995). Most platelet agonists induce phospholipase C activation and inositol trisphosphate (IP3) production leading to Ca^{2+} mobilization. This is important but not sufficient to switch on the scrambling process since an influx of Ca^{2+}, via store-operated Ca^{2+} entry (SOCE), appears essential (Kunzelmann-Marche et al. 2001). Recent reports implicate the SOCE machinery, including the Orai 1 channel and the STIM1 Ca^{2+} sensors, in PS exposure following collagen activation of mouse platelets (Bergmeier et al. 2009; Gilio et al. 2010). However, using STIM1- and Orai 1-deficient platelets, Gilio et al. (2010) found that another still uncharacterized mechanism of Ca^{2+} entry initiated by thrombin receptors also contributes to PS exposure.

The interactions between membrane glycoproteins and the cytoskeleton can also impact on the process of PS exposure. For instance, glycoprotein Ib-V-IX complex, the receptor for von Willebrand factor on platelets in flowing blood, modulates platelet procoagulant activity through the intracellular cytoskeleton-anchoring portions of GPIb (Ravanat et al. 2010). The calcium-dependent protease calpain, known to hydrolyze filamin A and spectrin, contributes to platelet

PS exposure (Comfurius et al. 1985; Fox et al. 1990). Of note, the physiological shear stress found in arteries significantly enhances agonist-induced platelet PS exposure in a small GTPase Rac1-dependent manner (Delaney et al. 2014). The lateral organization of plasma membrane and the dynamics of cholesterol and sphingolipid rich-microdomains also appear to be important for phospholipid scrambling (Kunzelmann-Marche et al. 2002).

PS exposure, resulting from inhibition of phospholipid translocase and activation of scramblase, is also a hallmark of apoptotic cells and a signal to eliminate these cells via phosphatidylserine receptors of professional phagocytes. Besides PS exposure, other features of strong platelet activation required for procoagulant activity resemble those occurring in apoptotic cells, including membrane blebbing and microparticle production. Platelets express many apoptotic regulators such as members of the Bcl-2 protein family, caspases, and antiapoptotic proteins such as BcL-x_L which maintain platelet viability (see Leytin (2012) for a review). It is proposed that two distinct molecular mechanisms, yet incompletely characterized, regulate platelet PS exposure induced either by apoptosis or by agonist-mediated platelet procoagulant function (Schoenwaelder et al. 2009). These data are consistent with a previous study demonstrating that, in Scott syndrome, PS exposure in apoptotic lymphocytes is normal, whereas calcium-induced PS exposure is absent in these cells (Bevers et al. 1992). Whether the role of PS exposure at the surface of apoptotic platelets can impact on thrombin generation in vivo or is exclusively a signal to eliminate these platelets remains to be established.

The Scott Syndrome

Scott syndrome is a rare inherited autosomal recessive bleeding disorder due to a defect in platelet procoagulant activity (Toti et al. 1996; Zwaal and Schroit 1997; Zwaal et al. 2004). It was first described by Weiss et al. (1979) in a patient with a history of bleeding episodes due to an impaired platelet procoagulant activity (Mrs M.A. Scott, 1939–1996). In the mid-1980s, Scott syndrome was recognized as a very rare, moderately severe bleeding disorder characterized by a defect of membrane phospholipid scrambling in activated platelets (Rosing et al. 1985b). The phospholipid composition of the outer plasma membrane leaflet of Scott syndrome platelets remains unchanged after activation by a mixture of thrombin *plus* collagen (Rosing et al. 1985b). Although the scrambling defect was also observed in red cells and lymphocytes (Bevers et al. 1992; Williamson et al. 2001), most patients do not experience clinical symptoms other than bleeding. The platelet count and structure are normal in Scott syndrome. Moreover, no significant defect of platelet secretion, aggregation, metabolism, granule content, platelet adhesion to subendothelium, or coagulation factor levels could be observed (Satta et al. 1997; Zwaal et al. 2004). The apparently unique defect of Scott syndrome platelets is the failure to promote fibrin formation in the presence of coagulation factors. Prothrombin consumption during clotting of whole blood is impaired and not restored by addition of normal plasma. Thus, measurement of residual prothrombin in serum is a simple laboratory screening test for the diagnosis of this syndrome. Diagnosis will be confirmed by flow cytometry, with annexin V binding on platelets (annexin V binds to PS in the presence of Ca^{2+}) indicating the lack of PS exposure following Scott syndrome platelet activation (Toti et al. 1996). A similar defect was also observed in a canine model presenting a deficiency in platelet procoagulant activity (Brooks et al. 2002, 2010).

Identification of TMEM16F as an Essential Component of the Calcium-Dependent Scramblase Activity Deficient in Scott Syndrome

The identity of the scramblase remained elusive until recently when Suzuki et al. identified the protein TMEM16F, also called anoctamin 6 (Ano6), a member of a family of Ca^{2+}-gated channel (Galietta 2009) as an essential component of the Ca^{2+}-dependent PS exposure (Suzuki et al. 2010). This discovery started with the isolation of a Ba/F3 cell subline exhibiting strong PS exposure in the absence of Ca^{2+} ionophore. This cell subline allowed identification of a cDNA encoding a constitutively active mutant of TMEM16F that was responsible for this effect. TMEM16F is a protein of predicted molecular mass of 106 kDa located on the plasma membrane with eight transmembrane domains. The TMEM16F expressed in the cell subline expressing PS constitutively exhibited a point mutation replacing the aspartate 409 by a glycine (D409G). Importantly, a sequence analysis indicated that the cDNA from a patient with Scott syndrome lacked the 226-bp sequence corresponding to exon 13 of the gene encoding TMEM16F. Direct sequencing of the chromosomal DNA showed that the TMEM16F gene of the patient carried a homozygous G-to-T mutation in the splice acceptor site in intron 12 causing exon 13 to be skipped. The resulting frameshift led to a premature termination of the protein at the third transmembrane domain of TMEM16F. Two novel mutations in the TMEM16F gene have been described in another patient with Scott syndrome (Castoldi et al. 2011) with previously characterized scramblase defect of his platelets (Munnix et al. 2003). The mutations were identified in intron 6 (G-to-A) disrupting the donor splice site consensus sequence and in exon 11 as a single-nucleotide insertion

predicting a frameshift and a premature termination of translation at codon 411. The first mutation is predicting the in-frame deletion of 38 amino acids in the N-terminal cytoplasmic tail of TMEM16F, and the second mutation is predicting the truncation of the protein between the second and the third transmembrane domains. Importantly, TMEM16F knockout mice recapitulate the platelet procoagulant defects that characterize Scott syndrome patients (Yang et al. 2012).

Following this breakthrough, a critical question remains: is TMEM16F a phospholipid scramblase itself? The literature provides very strong arguments that TMEM16F is a critical regulator of the Ca^{2+}-induced lipid scrambling processes, but, based on structural and biophysical data, it is difficult to conclude that this protein is indeed the scramblase itself (Lhermusier et al. 2011; Picollo et al. 2015). So far, a model explaining how TMEM16F could catalyze lipid movements by positioning adequately water molecules, lipid head groups, and the fatty acid chains is lacking. Thus, it is still controversial whether it is a channel, a scramblase, or both. Yang et al. showed that TMEM16F is a Ca^{2+}-activated channel permeable to Ca^{2+} and critical for the platelet scramblase activity (Yang et al. 2012). These authors propose that TMEM16F is essential but not sufficient for the Ca^{2+}-dependent scramblase activity that may be due to another protein. There are also contrasting results concerning the ion channel activity of TMEM16F (Suzuki et al. 2013). However, recent studies brought evidence that TMEM6F is both a Ca^{2+}-gated channel and a Ca^{2+}-dependent scramblase. Malvezzi et al. have shown that the ancestral TMEM16 homologue from *Aspergillus fumigatus* has the dual activity (Malvezzi et al. 2013). Moreover, by comparing the characteristics of TMEM16F variants generated by alternative splicing, Scuderi et al. also published data that bolster the idea that TMEM16F is directly involved in ion conduction and phospholipid scrambling (Scudieri et al. 2015). Some results argue with the fact that the two activities are not mutually exclusive (Malvezzi et al. 2013; Kmit et al. 2013; Yu et al. 2015). The recently published crystal structure of a TMEM16 family member (Brunner et al. 2014) provides further insight into the potential coexistence of the two activities: ion channels and phospholipid scramblase in the TMEM16 family. Based on these important but somehow conflicting results, three possibilities remain valid: (1) TMEM16F is a Ca^{2+}-dependent scramblase with no ion channel activity, (2) TMEM16F is a Ca^{2+}-dependent ion channel, and (3) TMEM16F possesses both intrinsic ion channel and scramblase activities.

Further studies are required to unambiguously characterize the intrinsic function of TMEM16F. The molecular interplay between lipid scrambling and ion channels remains to be fully elucidated, leaving still open the long lasting and fascinating story of scramblase identification in platelets.

Platelet Procoagulant Function in Pathology

Besides the very rare inherited Scott syndrome, due to a primary defect in platelet procoagulant activity, abnormal ability of platelets to express negatively charged phospholipids upon activation with physiological agonists seems to be relatively frequent. In vitro, strong stimulation of platelets with a combination of thrombin and collagen has led to the characterization of a fraction of platelets exposing on their surface, not only PS but also various proteins secreted from α-granules (Dale 2005). They have been named "coated platelets" because their surface is coated with adhesive and procoagulant proteins. They are also called "superactivated" platelets (Mazepa et al. 2013) because they exhibit a stronger ability to promote thrombin generation. They can be detected by flow cytometry measurement of Annexin-V binding and co-staining of factor V/Va. Confocal microscopy analysis has revealed that the secreted α-granule protein coat organizes into a cap resembling a small ball-like structure polarized to a distinct zone (Abaeva et al. 2013). Coated platelets are virtually absent in unstimulated platelets (<2 % of the platelet population), but this proportion rises to about 30 % after stimulation (Alberio et al. 2000) with a large variability (15–50 %) between healthy subjects (Dale 2005). What explains such platelet heterogeneity, besides their younger age in circulation, and why such variability in response between subjects, remain largely unknown. In a recent study (Daskalakis et al. 2014), isolated impairment of the ability to generate coated platelets in vitro (<20 % of cells converted into coated platelets) was identified in a significant number of patients with mild bleeding diathesis and non-diagnostic laboratory work-up. Of note, these patients had a normal expression of negatively charged phospholipids in response to the calcium ionophore A23187. The correlation between the ability to form coated platelets following stimulation and the patient symptoms has been studied. The results indicate lower levels of coated platelet formation in hemorrhagic conditions. Conversely, higher levels were found in ischemic strokes and related diseases, suggesting a contribution of coated platelets to thrombosis in the arterial and microcirculation (Daskalakis et al. 2014).

Moreover, in patients with primary platelet disorders and impaired aggregation response to various agonists, the administration of desmopressin (DDAVP) enhanced the ability to form coated platelets and subsequent thrombin generation, in parallel with sustained free cytosolic Ca^{2+} increase in platelets. This observation provides a possible mechanism of desmopressin action, independent from its effect on von Willebrand factor and factor VIII release (Colucci et al. 2014).

Taken together, these results suggest that the platelet procoagulant activity elicited by physiological agonists is

supported, at least in vitro, by a subset of circulating platelets. The ability to produce such "super-platelets" (Pecci and Balduini 2014) is variable between subjects, but so far only limited data suggest their role as potential clinical or pharmacological target.

Platelet Fibrinolytic Functions

Fibrinolysis and Platelet Interplay

The main function of fibrinolysis is the dissolution of fibrin clots in blood vessels. The fibrinolytic system comprises a proenzyme, plasminogen, which can be converted into the active serine protease plasmin by two main plasminogen activators: the tissue-type plasminogen activator (t-PA) and the urokinase-type plasminogen activator (u-PA). Inhibition of fibrinolysis occurs at the level of these activators by plasminogen activator inhibitors (PAIs) or at the level of plasmin, mainly by the alpha 2-antiplasmin (α2AP). Proteolytic cleavage of fibrin by plasmin generates C-terminal lysine residues capable of binding both plasminogen and t-PA, thereby propagating fibrinolysis. This positive feedback mechanism is negatively regulated by activated thrombin-activatable fibrinolysis inhibitor (TAFI), which cleaves C-terminal lysine residues from the fibrin surface. Currently, administered thrombolytic drugs consist of recombinant PAs that rapidly lyse thrombi by catalyzing the formation of plasmin (Longstaff and Kolev 2015).

Platelets and the fibrinolytic system interact in several ways (Fig. 2). We will discuss here some clinically relevant aspects of these complex interrelations. It is now clear that platelets can modulate endogenous fibrinolysis and also the outcome of thrombolytic therapy by delaying reperfusion and mediating reocclusion and also by promoting bleeding complications (Coller 1990; Pasche and Loscalzo 1991). Therefore, it is worthwhile to understand the different facets of the relationship between platelets and fibrinolysis. Moreover, it is noteworthy that a rare platelet pathology, the Quebec platelet disorder, is a unique gain-of-function defect in platelet fibrinolysis due to an increase in u-PA production by megakaryocytes and storage in platelet granules (Blavignac et al. 2011).

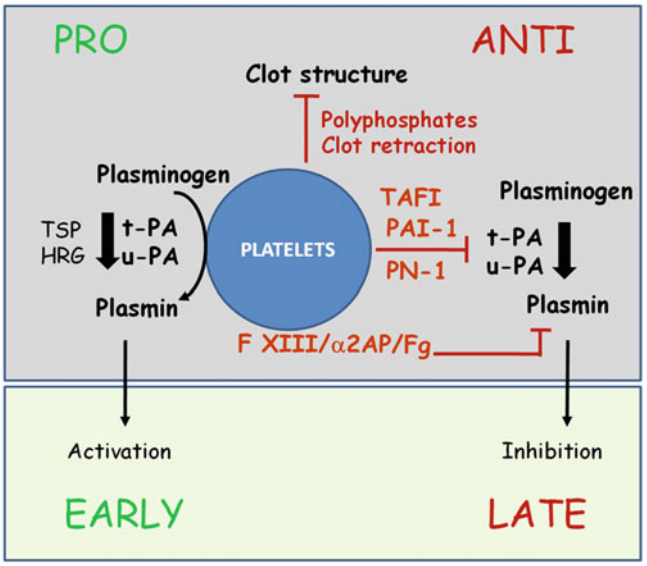

Fig. 2 Platelets and fibrinolysis: a schematic representation of the interplay between platelets and fibrinolysis. *Upper panel*: Profibrinolytic activity (PRO) involved the binding of plasminogen and u-PA at the cell surface and the release of α-granule proteins [thrombospondin (TSP), histidine-rich glycoprotein (HRG)]. Antifibrinolytic properties involved the release of fibrinolysis inhibitors (TAFI, PAI-1, PN-1). Polyphosphates and clot retraction impair fibrinolysis through modification of clot structure. Platelet factor XIII (FXIII) participates to the fibrinolysis inhibition by mediating cross-linking between platelet α2AP and platelet fibrin(ogen) (Fg). *Lower panel*: During the early phase of thrombolysis, the platelets become transiently activated and, consequently, delay the lytic process. In the late phase, however, platelets become progressively inhibited

Platelets in Fibrinolysis Resistance

Studies mimicking the physiopathological conditions have consistently reported that platelets play a pivotal role in fibrinolysis resistance (Jang et al. 1989; Fay et al. 1994) and that antiplatelet agents enhance the efficacy of thrombolytic treatment (Collet et al. 2001; Yasuda et al. 1990, 1991; Fitzgerald and Fitzgerald 1989; Lam et al. 1991; Ohman et al. 1997). Both in vitro and in vivo data point to different mechanisms of platelet-mediated fibrinolysis resistance.

Cellular factor XIII (XIIIa) is abundant in platelets and is thought to play a major role in lysis resistance. Analysis of plasma clot structure by polyacrylamide gel electrophoresis and immunoblotting revealed accelerated α2AP-fibrin cross-linking in platelet-rich compared with platelet-depleted plasma clots (Reed et al. 1991). In washed platelets aggregated by thrombin, a platelet factor XIII-mediated cross-linking between platelet α2AP and platelet fibrin(ogen) has been observed. Using the Chandler model, it has been shown that thrombi formed from factor XIII-depleted plasma are stabilized by platelets in a factor XIII-dependent manner (Mitchell et al. 2014). Incorporating a neutralizing antibody to α2AP into factor XIII-depleted thrombi revealed that the stabilizing effect of platelet factor XIIIa on lysis was dependent on α2AP. Fluorescence confocal microscopy and flow cytometry studies have unveiled the exposure of factor XIIIa on activated platelet membranes, with maximal signal detected with thrombin and collagen stimulation, which is also known to induce coated-platelets formation. Factor XIIIa was evident in protruding caps on the surface of PS-positive platelets.

This indicates that the subpopulation of coated platelets (see Kasirer-Friede and Shattil 2017) displays both procoagulant and antifibrinolytic properties.

Release of fibrinolysis inhibitors by activated platelets also makes clots resistant to fibrinolysis. PAI-1 is present in platelet α-granules. A large fraction (92 %) of PAI-1 antigen is released from platelets, whereas only 8 % is found in the corresponding platelet-poor plasma. The bulk of PAI-1 released from platelets is inactive, but it is unclear whether this inactive material is already present in platelets or whether platelet PAI-1 is inactivated during release (Declerck et al. 1988). Several studies are in favor of a functional role of platelet PAI-1 and underline its contribution in fibrinolysis resistance. When washed human platelets are added to a t-PA-dependent lysis system, the clot lysis becomes highly sensitive to the addition of thrombin. De Fouw et al. (1987) reported a more than threefold increase in the time necessary to reach half maximal lysis when the thrombin concentration was raised from 0.5 to 12.2 nM in the presence of 0.2×10^9 platelets/mL. The explanation given for this platelet-dependent inhibition of clot lysis is that thrombin-activated platelets release PAI-1 which in turn neutralizes a part or possibly all t-PA present in the system. Keijer et al. (1991) further demonstrated that addition of a monoclonal antibody against PAI-1, which inhibits t-PA neutralization, completely quenches the inhibitory effect of platelets on t-PA-dependent clot lysis in a system of purified components. Together these experiments indicate that, locally, the time course of thrombin formation and the availability of platelets are important parameters in determining the lysability of thrombi. These in vitro results were confirmed by the studies performed by other groups (Braaten et al. 1993; Booth et al. 1992; Stringer et al. 1994). Experiments conducted in vivo showed that platelet-dependent resistance could be completely neutralized by anti PAI-1 molecules (Levi et al. 1992; Rupin et al. 2001). The effect of platelet PAI-1 on fibrinolysis in humans is supported by the severe autosomal recessive bleeding disorder described in patients with complete loss of PAI-1 in plasma and platelets (Fay et al. 1992).

Thrombin activatable fibrinolysis inhibitor (TAFI) is a fibrinolysis inhibitor that provides a balance between coagulation and fibrinolysis. TAFI is activated by thrombin to yield an active carboxypeptidase, TAFIa. TAFIa regulates fibrinolysis by removing carboxyterminal lysine residues from fibrin. These lysine residues are exposed during plasmin degradation of fibrin and are important for the binding of plasminogen and t-PA and fibrinolysis amplification. Removal of these sites by TAFIa prolongs the rate of lysis. The presence of TAFI in platelet granules has been established in 2003 (Mosnier et al. 2003). It was first observed that TAFI from platelets contributes less than PAI-1 to fibrinolysis resistance of platelet-rich clots (Mutch et al. 2007). However, using thromboelastography, Carrieri et al. (2011) have shown that neutralization of TAFI, but not of PAI-1, shortened the lysis time suggesting that TAFI activation is a major mechanism whereby platelets make clots resistant to fibrinolysis.

Protease nexin-1 (PN-1) is a serpin that inhibits plasminogen activators, plasmin and thrombin, and is present within the α-granules of platelets but barely detected in plasma. PN-1 deficiency within platelets results in increased clot lysis. Vascular recanalization is significantly increased in PN-1-deficient mice suggesting that PN-1 inhibition promotes endogenous and exogenous t-PA-mediated fibrinolysis (Boulaftali et al. 2011).

It is noteworthy that in addition to favoring the release of fibrinolysis inhibitors from platelets, clot retraction has been demonstrated to affect clot structure and t-PA binding and diffusion (Kunitada et al. 1992; Collet et al. 2001, 2002; Katori et al. 2005; Weisel and Litvinov 2008) contributing to fibrinolysis resistance. Moreover, platelets are a source of polyphosphate, which is released from activated platelets and supports localized changes in fibrin structure. Polyphosphates of sufficient length have been shown to result in thicker fibrin strands that are more resistant to fibrinolysis (Mutch et al. 2010).

Contribution of Platelets to Fibrinolysis

Besides being implicated in fibrinolysis resistance, platelets have also been shown to promote fibrinolysis. On their membrane, platelets can bind plasminogen (Miles and Plow 1985) and express urokinase receptors (Wohn et al. 1997; Jiang et al. 1996). Remarkably, plasminogen binds platelets by two distinct mechanisms: (1) a direct binding and (2) a fibrin(ogen)-dependent binding on PS-exposing platelets (Whyte et al. 2015). This relatively late mechanism suggests that the subpopulation of procoagulant coated platelets retain on their cap not only procoagulant and antifibrinolytic but also profibrinolytic factors. Platelets, like many cells, provide a surface for enhanced plasminogen activation by promoting the interaction between t-PA and plasminogen (Deguchi et al. 1985; Ouimet et al. 1994) and by enhancing clot lysis mediated by single chain u-PA (Baeten et al. 2010; Loza et al. 1994; Lenich et al. 1997).

In addition, secreted α-granule proteins may contribute to fibrinolysis. Significant amounts of plasmin can be generated from the trimolecular complex formed between the granular protein thrombospondin, histidine-rich glycoprotein, and plasminogen (Silverstein et al. 1985).

These data indicate that platelets exhibit pro- and antifibrinolytic effects (Fig. 2). This balance may be regulated by the level of platelet activation and secretion and may be different at the cell surface and in the platelet environment.

Impact of Fibrinolysis/Thrombolysis on Platelet Function

The success of PAs in recanalizing occluded coronary arteries may be influenced by their effect on blood platelets. Upon exposure to a fibrinolytic milieu, platelets become transiently activated and, consequently, delay the progression of the lytic process. However, following continued exposure to a fibrinolytic milieu, platelets become progressively inhibited, and, as a result, bleeding times prolong and hemorrhagic risk increases. As described below, the molecular events leading to these changes in platelet function during thrombolysis are complex. Increased plasmin and plasminogen activator levels during thrombolysis can cause platelet activation and aggregation. When added to platelets, plasmin can induce aggregation (Puri et al. 1992) and a prompt, concentration-dependent $[Ca^{2+}]i$ increase and 5-HT release (Penny and Ware 1992). Plasminogen activators can increase platelet surface P-selectin expression in a concentration-dependent manner (Kawano et al. 1998). These platelet effects occur at clinically achievable concentrations and may affect the success of therapy with thrombolytic agents. Such activation is followed by subsequent inhibition of cellular activation by a second agonist; this inhibitory effect being in proportion to the degree of initial activation and ADP is an important cofactor in both processes (Penny and Ware 1992). This second wave of platelet inhibition has been mainly attributed to GPIb and GPIIbIIIa degradation (Stricker et al. 1986; de Haan and van Oeveren 1998; Kamat et al. 1995). A study in patients treated with 150 mg of rt-PA given over 6 hours has suggested a redistribution of GPIb induced by plasmin with an increased circulating level of glycocalicin, a proteolytic fragment of GPIbα, and a concomitant decrease in whole platelet GPIb content but without change in GPIb surface expression (Michelson et al. 1990). Moreover, administration of the nonspecific protease inhibitor with antifibrinolytic activity, aprotinin, increases the number of surface GPIb platelet receptors in patients who bled in excess during cardiac surgery (Kallis et al. 1994). Also, the antifibrinolytic tranexamic acid shortens the bleeding time and improves platelet aggregation and secretion in patients with chronic renal failure (Mezzano et al. 1999).

Overall, the strong interplay evidenced between platelets and fibrinolysis may contribute to the control of the bleeding/thrombosis balance during thrombolysis and in a large set of clinical conditions associated with fibrinolysis activation.

Quebec Platelet Disorder

Quebec platelet disorder (QPD) is a very rare autosomal dominant bleeding disorder associated with a unique gain-of-function defect in platelet fibrinolysis (see Hayward and Rivard (2011) for a comprehensive review). It was initially reported in a family of patients with delayed, moderate to severe bleeding, responsive to fibrinolytic inhibitors, and first attributed to a deficiency in platelet factor V, hence called factor V Quebec in 1984 (Tracy et al. 1984). The name was changed to Quebec platelet disorder with the description of α-granule protein proteolysis, abnormal aggregation response to various agonists, and moderately reduced platelet count (Hayward et al. 1996, 1997).

The biochemical defect of QPD and the pathogenesis were elucidated 15 years after the first report, as being a massive increase in the α-granule stores of u-PA. This increase in u-PA triggers the intra-platelet generation of plasmin from the plasminogen endocytosed into α-granules in circulating platelets and the degradation of stored proteins (Sheth et al. 2003; Kahr et al. 2001). As developed in Colucci et al. (2017), when activated, normal platelets display a subtle profibrinolytic activity as a secondary process to their procoagulant activity (Kim 2015), tightly controlled by the natural inhibitors (PAI-1, α2-antiplasmin, TAFI) released from the α-granules. The degradation of these regulators and the release of large amounts of u-PA and plasmin in the growing thrombus explain the instability and premature lysis of the clot and thereby the bleeding phenotype and its exquisite sensitivity to fibrinolytic inhibitors.

The molecular origin of QPD was demonstrated to be a massively increased expression of the u-PA gene (*PLAU*) in differentiated megakaryocytes (Diamandis et al. 2009; Veljkovic et al. 2009), without change in the expression of the other platelet proteins. The unique QPD mutation, described in all unrelated families so far, is a direct tandem duplication of a large 77 kb region on chromosome 10 that includes *PLAU* and its regulatory elements, in addition to the gene of unknown function, *c10orf55* (antisense to *PLAU*) (Paterson et al. 2010). To date, QPD is the only inherited bleeding disorder known to be caused by a *PLAU* mutation and the only one attributed to a gene duplication mutation. How only one extra copy of *PLAU* can cause more than 100-fold increase in u-PA production by megakaryocytes is unknown. Why this mutation does not result in the overproduction of u-PA in other tissues, since plasma and urinary u-PA levels are normal in QPD (Diamandis et al. 2008), remains also unexplained.

The clinical features of QPD consist in delayed bleeding (1–3 days) following hemostatic challenges, easy bruising, prolonged bleeding after deep cuts, muscle and joint bleeds, hematuria, long menses, and postpartum hemorrhages (McKay et al. 2004; Blavignac et al. 2011). The bleeding scores of affected patients are easily distinguished from those of their unaffected relatives (McKay et al. 2004).

Apart from bleeding symptoms, there are no other known clinical manifestations of QPD (Tracy et al. 1984; McKay et al. 2004). There are no reproductive disorders and, strikingly, QPD does not preclude thromboembolic events. Most laboratory findings of current practice, such as moderate thrombocytopenia with normal platelet and megakaryocyte morphology, low factor V, and reduced aggregation response to epinephrine, ADP, or collagen, are nonspecific of QPD. A simple screening test is the measurement of D-dimer levels, unusually high in the serum from clots prepared from whole blood compared to platelet-poor plasma (Diamandis et al. 2006). The confirmatory test consists in the detection of increased u-PA and degraded forms of α-granule proteins in QPD platelets by Western blot assays and antigen assays to quantify u-PA. Finally, the method of choice for the diagnosis of QPD is the detection by PCR assay of the *PLAU* gene duplication mutation (Paterson et al. 2010). Although a founder effect is likely from the epidemiology and genetics of QPD, it cannot be formally excluded that QPD-like disorders can be caused by other *PLAU* mutations.

The treatment of QPD is fibrinolytic inhibitor therapy at standard doses. Desmopressin and platelet transfusions are ineffective (Hayward et al. 2006).

Conclusion

Activated platelets are considered the prominent cell membrane surface required for the assembly of coagulation factor complexes and the formation of the fibrin network. In addition, they also contribute to modulate fibrinolysis. However, much of our current understanding of the platelets' contribution to coagulation and fibrinolysis derives from in vitro/ex vivo studies that may not reliably reflect hemostatic events under the influence of the native environment. Therefore, many important questions remain open such as:

1. How is the spatiotemporal regulation of platelet coagulant and fibrinolytic responses organized?
2. What is the in vivo implication and functions of the coated platelets?
3. What is the relative contribution of platelets and fibrin surfaces to fibrinolysis?
4. How is the distribution of procoagulant or fibrinolytic platelet surfaces altered in pathological conditions resulting either in hemorrhage or thrombosis?

The remarkable advances in intravital imaging techniques in the last decade should make it possible to answer these questions in the near future.

Take Home Messages

- Platelet procoagulant activity is triggered by a calcium-dependent scramblase activity, allowing exposure of the anionic phospholipid, phosphatidylserine, at the plasma membrane surface.
- TMEM16F is a membrane protein essential for calcium-dependent phosphatidylserine exposure with loss of function mutations in Scott syndrome.
- Platelets have the capacity to interfere with the fibrinolytic system in several ways and by doing so they can modulate the outcome of thrombolytic therapy.
- The Quebec platelet disorder is a rare autosomal dominant bleeding disorder characterized by a unique gain-of-function defect in fibrinolysis.

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Acquired Disorders of Platelet Function

Rüdiger E. Scharf

Abstract

Platelet function can be abnormally *increased*, as in association with vascular events, or *defective*, as in a variety of clinical settings, or display converse phenotypes with *increased* and *decreased* platelet responsiveness, as in myeloproliferative neoplasms. Drugs represent the most common cause of platelet dysfunction in our overmedicated society. While aspirin, clopidogrel, prasugrel (or more recently also ticagrelor), and integrin $\alpha_{IIb}\beta_3$ receptor antagonists (abciximab, eptifibatide, and tirofiban) are well-known prototypes of antiplatelet drugs, other widely used agents (e.g., nonsteroidal anti-inflammatory drugs, antibiotics, serotonin reuptake inhibitors, and volume expanders) and supplements can also impair platelet function and thus cause or aggravate hemorrhages. Apart from pharmacological agents, certain clinical conditions are often associated with qualitative platelet disorders and a bleeding diathesis. Consequently, in contrast to inherited platelet disorders, acquired platelet function defects are much more frequent in clinical practice and therefore deserve special attention. Their pathogenesis is widespread and heterogeneous with various, sometimes overlapping abnormalities in the same clinical setting. Moreover, acquired platelet dysfunction may occur at any age, and platelet defects can range in severity from mild to life-threatening hemorrhages. Diagnostic work-up of platelet disorders requires meticulous evaluation of medical history, specifically of any drugs interfering with platelet function, careful clinical examination, and a staged laboratory protocol to assess the underlying platelet defect(s). Interpretation of laboratory findings may sometimes be difficult, as reduced platelet responsiveness obtained by in vitro settings may result from "exhausted" platelets due to increased activation in vivo. To identify hyperactive platelets ex vivo, costly procedures are required using flow cytometry in combination with specific monoclonal antibodies. Currently, this approach can be recommended for research purposes only. Platelet dysfunction, sometimes in association with quantitative abnormalities, is present in uremia, liver disease, hematologic disorders, cardiac valvular disease, and extracorporeal settings. Due to their heterogeneity, acquired platelet function disorders will be discussed according to the underlying clinical condition.

Introduction

Platelets contribute essentially to survey the integrity of the vascular system. They respond immediately to vascular lesions by becoming adherent within milliseconds and by forming aggregates at sites of injured endothelial cells or exposed subendothelial matrix structures. Following activation,

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platelets provide a highly effective catalytic membrane surface for the generation of thrombin that in turn accelerates the recruitment of circulating platelets and the formation of fibrin necessary to stabilize thrombi and to prevent their detachment by flowing blood (Ruggeri 2009). Once stimulated, platelets respond uniformly and do not distinguish between traumatic injury and atherosclerotic or inflammatory damage of the vessel wall (Ruggeri and Mendolicchio 2007, 2015). While their physiological function is to support arrest of bleeding, to contribute to host defense and wound healing, and to restore vessel wall integrity, platelets can form occlusive thrombi as a consequence of vascular diseases, such as atherosclerosis. Thus, under pathological conditions, platelet responses may result in acute ischemic syndromes of the heart, brain, and other organ systems.

Antiplatelet agents are therefore being widely used to prevent and treat thromboembolic events in patients with arterial disease (Aguilar and Hart 2005; Antithrombotic Trialists' Collaboration 2002, 2009). Overall, it has been documented that the risk of severe bleeding increases, when more intense antithrombotic regimens are administered (Eikelboom and Hirsh 2007; May et al. 2008). This is especially true in comorbid patients with preexisting hemostatic defects of any kind. Such disorders may remain compensated, unless platelet function is not inhibited pharmacologically (Scharf 2009) (Box 1). Apart from well-known prototypes of antiplatelet agents such as acetylsalicylic acid, thienopyridines, and integrin $\alpha_{IIb}\beta_3$ receptor antagonists, other widely used agents and supplements can impair platelet function (Scharf 2012) and, consequently, cause or aggravate hemorrhages in certain conditions (Scharf 2009).

This chapter will consider both features of platelet abnormalities, namely, platelet hyperreactivity (associated with a prothrombotic state) and, conversely, defective platelet function that occurs in a variety of clinical settings or is due to pharmacological inhibition, or both. Relevant developments are reviewed, and recent insights into pathomechanisms will be discussed.

Increased Platelet Function

Platelet Hyperreactivity and Prothrombotic State(s)

Numerous experimental and clinical studies have documented that vascular events cause platelet activation and formation of platelet thrombi, which in turn can lead to acute coronary syndromes, myocardial infarction, or stroke (Fitzgerald et al. 1986; Scharf 1986a, b; Fuster et al. 1992). Thus, platelet activation occurs at the site of a fissured coronary atherosclerotic plaque with exposure of highly thrombogenic substances (Fuster et al. 1992; Ruggeri and Mendolicchio 2015).

For example, the effect of angioplasty-injured coronary arteries on platelet activation has been studied (Scharf et al. 1992). Using flow cytometry in combination with specific monoclonal antibodies, activated platelets were demonstrated in patients undergoing percutaneous transluminal coronary angioplasty (Fig. 1). By contrast to these conditions, in which platelet activation and thrombus formation emerge in response to the exposure of subendothelial extracellular matrix proteins, less well documented, and perhaps less frequent, are several clinical settings, in which an acute vascular event appears to result from intrinsic platelet activation. Patients with transient neurological deficits in migraine accompagnée provide a striking example of this assumption (Scharf et al. 1982, 1983a). In this setting, platelet activation markers can correlate inversely with the interval between the time of a migraine attack and blood collection (Fig. 2), suggesting a direct association of increased platelet secretion with clinical symptoms. This observation is in accord with findings from others, who reported on an augmented rate of circulating platelet aggregates in patients with migraine accompagnée at the time of migraine attacks (Hannington et al. 1981).

Along with suchlike findings, it has been postulated clinically that hyperactive platelets exist (Davies and McNicol 1981; Yee et al. 2005). From a laboratory point of view, this condition has been termed platelet hyperaggregability, defined as a feature, in which the threshold concentration for aggregating agents, including ADP, epinephrine, and collagen, is lowered in patients (Trip et al. 1990; Yee et al. 2005)

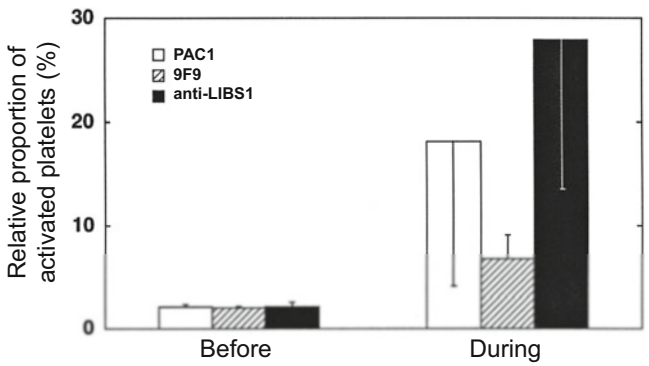


Fig. 1 Flow cytometric detection of circulating activated platelets in patients undergoing percutaneous transluminal coronary angioplasty (PTCA). Blood was sampled continuously via a heparin-coated catheter placed in the coronary sinus before vascular intervention and remained in situ 30 min after termination of PTCA to allow further collection of blood samples. Binding of fluoresceinated monoclonal antibodies specific for activation-dependent platelet epitopes, including activated $\alpha_{IIb}\beta_3$ (PAC-1), fibrinogen bound to $\alpha_{IIb}\beta_3$ (9F9), ligand-induced binding sites on β3 (anti-LIBS1), and P-selectin (S12), was evaluated by flow cytometry. A 2 % platelet subpopulation with the brightest membrane fluorescence intensity at baseline was analyzed separately and quantified for relative increase in platelet number during PTCA. Bars represent mean ± SD. *P*-values for increased binding of PAC-1, anti-LIBS1, and 9F9 were <0.01, <0.01, and 0.03, respectively. Adapted from Scharf et al. (1992) and reprinted with permission of the publisher

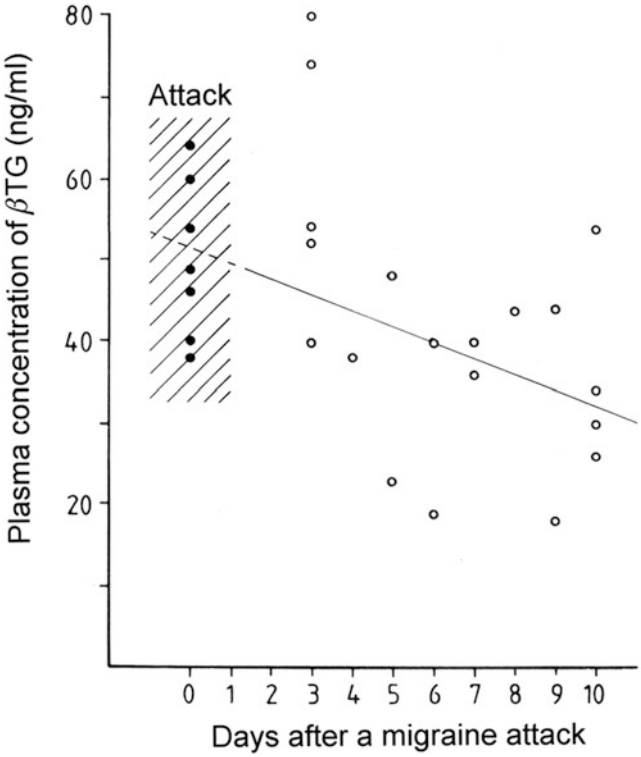


Fig. 2 Platelet secretion in patients with migraine acompagnée. Blood was collected at the time of a migraine attack (*hatched area*) in seven patients (*solid circles*) and at various days after a migraine attack in 19 patients (*open circles*). β-thromboglobulin (βTG) was determined in platelet-free plasma as a marker of platelet secretion in vivo. Note the inverse relationship of the individual plasma levels of βTG and the interval between time of migraine attacks and blood collection ($r = -0.7211$, $p < 005$). Adapted from Scharf (1986a) and reprinted with permission of the publisher

and also in healthy individuals (Yee et al. 2005). This phenomenon should be carefully distinguished from the "sticky platelet syndrome," an autosomal dominant hereditary disorder (Mammen 1999). In a more general way, the term "prothrombotic state" has been introduced. It is postulated for a condition, which precedes clinically overt thrombosis during which hemostasis is altered in a way that promotes formation or deposition of platelet thrombi and generation of fibrin (Davies and McNicol 1981). This definition has raised several questions, including suitable biomarkers of a prothrombotic state and its molecular nature. In this context, distinct polymorphisms of platelet membrane glycoprotein receptors have come into the focus of interest.

Polymorphisms of Platelet Membrane Glycoproteins (GP)

Platelet membrane GP are highly polymorphic and can be recognized as self-antigens or alloantigens. Incompatibility of distinct epitopes, also known as human platelet antigens (HPA), on various platelet receptors is responsible for alloimmune thrombocytopenias and refractory platelet transfusions.

HPA-1 Polymorphism of $\alpha_{IIb}\beta_3$ Platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) carries HPA-1 and other diallelic alloantigen systems (Kunicki and Newman 1992). The HPA-1 polymorphism arises from a single T→C nucleotide substitution at position 1565 in the β3 gene, which in turn leads to an amino acid change at position 33 in the β3 subunit (GPIIIa) with leucine in HPA-1a (Leu33) and proline in HPA-1b (Pro33) (Newman et al. 1989). Importantly, the HPA-1b allele is not rare; approximately 25 % of Caucasians have at least one allele (Kunicki and Newman 1992).

Polymorphisms of $\alpha_2\beta_1$ Platelets contain several receptors for collagen, including GPVI and integrin $\alpha_2\beta_1$ (GPIa-IIa), both of which are important for platelet adhesion. $\alpha_2\beta_1$ exhibits at least three alleles of the α2 gene, which are defined by eight nucleotide polymorphisms (Kunicki et al. 1997). This integrin is expressed at low density on the platelet surface (1000–3000 receptor copies per platelet). Interestingly, there is an approximately tenfold variation among normal individuals that can modulate platelet responses to collagen (Kritzik et al. 1998). Allele 1 (807T/837T/873A/1648G), also referred to as $\alpha_2$807T, is associated with increased surface expression of $\alpha_2\beta_1$ (high-density variant), while alleles 2 (807C/837T/873G/1648G) and 3 (807C/837C/873G/1648A), also designated $\alpha_2$807C, are associated with low surface expression of the receptor (Kritzik et al. 1998). Indeed, in whole blood, the rate of platelet adhesion onto type I collagen under high-shear conditions is proportional to the density of $\alpha_2\beta_1$ receptor copies on the platelet surface (Kritzik et al. 1998). The frequency of allele 1 and 2 is 39 % and 53 %, respectively, and that of allele 3 is 7 % (Kunicki et al. 1997).

Prothrombotic Platelet Receptor Variants

Clinical Association Studies Weiss et al. (1996) first reported on an association between HPA-1b and myocardial infarction (MI). The prevalence of HPA-1b was higher in MI patients than in those without coronary artery disease (CAD) (39.4 % vs. 19.1 %, odds ratio 2.8, $p = 0.01$). In patients, whose MI occurred prior to the age of 60 years, an even greater difference between cases and controls (50 % vs. 13.9 %, odds ratio 6.2, $p = 0.002$) was observed. By contrast, no such difference in the prevalence of HPA-1b was documented in the Physicians' Health Study, prospectively comparing MI patients and matched individuals without cardiovascular events (25.2 % vs. 26.4 %, $p = 0.4$)

(Ridker et al. 1997). These two studies are representative for discrepant results published subsequently (Bray 2000).

Since then, the conflicting findings have been a matter of debate. The same is true for the α_2C807T polymorphism of integrin $\alpha_2\beta_1$. Moshfegh et al. (1999) found a higher prevalence of the $\alpha_2$807TT genotype in patients with MI as compared to healthy controls (16.4 % vs. 2.6 %, $p = 0.022$). By contrast, no significant association of the $\alpha_2\beta_1$ receptor variant and MI was observed by Santoso et al. (1999). However, in the same study, subgroups of younger patients (<62 or <49 years) revealed an increased risk in carriers of the T-allele (odds ratio 1.57, $p = 0.004$, or 2.61, $p = 0.009$, respectively).

In a retrospective study on patients with MI, those with CAD but no history of MI, and control patients, who all had undergone coronary angiography, Zotz et al. (1998) found no difference in the overall prevalence of HPA-1b among case patients with MI (23 %) and control patients (25 %, $p = 0.75$), confirming the results of the Physicians' Health Study (Ridker et al. 1997). However, a post hoc analysis revealed that the prevalence of HPA-1b was related to the patients' age and time after MI with highest values in MI patients <60 years of age and those with recent-onset (<1 year) MI (45 % vs. 23 % in controls, odds ratio 2.0, $p = 0.007$), thereby confirming the observations by Weiss et al. (1996). Moreover, patients with CAD, who are carriers of the HPA-1b allele, experienced a MI earlier in life than HPA-1b-negative patients (Zotz et al. 1998). The authors concluded that the HPA-1b allele of $\alpha_{IIb}\beta_3$ is not a risk factor for atherosclerosis but a risk factor for arterial thrombosis. Moreover, Zotz et al. (1998) hypothesized that HPA-1b is a prothrombotic risk determinant that requires the presence of an atherosclerotic lesion to become effective. Thus, unlike conventional risk factors, HPA-1b is not a risk factor for CAD as such but appears to be associated with increased platelet thrombogenicity. This contention provides an explanation for conflicting results of association studies on the role of the HPA-1 polymorphism of $\alpha_{IIb}\beta_3$ in CAD. Along with this hypothesis, it can be anticipated that, even in prospective studies, the effect of a polymorphism coupled to the presence of CAD will provide no difference in the prevalence of HPA-1b between MI patients and controls, since the receptor variant is not associated with an increased rate of MI but a *premature* onset of MI.

This hypothesis was tested in a cohort of 3250 carefully characterized patients, including survivors of MI, patients with CAD but no history of MI, and control patients (no angiographically detectable CAD) in comparison to healthy volunteers (Zotz et al. 2005). In a *case-control* design, the prevalence of HPA-1b and $\alpha_2$807TT genotypes did not differ between patients with CAD or MI and patient controls or healthy volunteers. By contrast, using a multivariate *case-only* design, the median age at onset of MI was 5.2 years earlier ($p = 0.006$) in carriers of the HPA-1b allele and 6.3 years earlier ($p = 0.006$) in carriers of the $\alpha_2$807TT genotype in 264 survivors of recent-onset MI (Fig. 3).

A prospective study on patients undergoing saphenous-vein coronary artery bypass grafting documented that the prevalence of HPA-1b was higher among those with bypass occlusion, MI, or death >30 days after surgery as compared to those without postoperative complications (60 % vs. 24 %, odds ratio 4.7, $p < 0.05$). Using a stepwise logistic regression analysis with the variables HPA-1b, age, sex, BMI, smoking, hypertension, diabetes, and hyperlipidemia, only HPA-1b had an association with bypass occlusion, MI, or death after bypass surgery (odds ratio 4.7, $p = 0.019$) (Zotz et al. 2000).

The results suggest that distinct integrin genotypes may modulate platelet function and lead to increased platelet thrombogenicity. As the effects of the critical receptor variants are coupled to the presence of CAD, it is likely that an atherosclerotic lesion (e.g., plaque instability) becomes a decisive trigger. The remarkable similarity, in which the genetically unlinked markers behave, supports the contention that HPA-1b (Pro33) of $\alpha_{IIb}\beta_3$ and $\alpha_2$807TT of $\alpha_2\beta_1$ are prothrombotic (Box 2).

Functional Properties of Platelet Integrin Variants Under Arterial Flow Conditions To define the genotype-phenotype relation of critical $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ variants, HPA-1b (Pro33) or $\alpha_2$807TT platelets were studied in an established model, simulating arterial flow conditions in combination with digital imaging. This model permits the analysis of shear-induced platelet adhesion and thrombus formation onto thrombogenic surfaces (Ruggeri 2009). Using these techniques, it was demonstrated that the HPA-1b variant of $\alpha_{IIb}\beta_3$ and the $\alpha_2$807TT genotype of $\alpha_2\beta_1$ have indeed prothrombotic properties. Major characteristics of their phenotype are increased adhesion activity (Loncar et al. 2007), increased thrombus formation (Scharf et al. 2009), and increased outside-in signaling (Scharf et al. 2009; Gyenes et al. 2011). These findings were confirmed with transfected cell lines expressing $\alpha_{IIb}\beta_3$ of either isoform, HPA-1a or HPA-1b. Upon exposure of adherent cells to increasing shear rates (up to 1600 s^{-1}), HPA-1b (Pro33) cells were more resistant than HPA-1a (Leu33) cells (El Khattouti et al. 2011). Along with these findings, it was concluded that the increased thrombogenicity of the Pro33 variant of $\alpha_{IIb}\beta_3$ is due to elevated thrombus stability resulting from a polymorphism-mediated modulation in mechanotransduction. Thus, thrombus formation and thrombus stability would be driven, at least in vitro, by the biophysical transmission of receptor-ligand interactions to the platelet cytoskeleton. In vivo, the prothrombotic character of HPA-1b (Pro33) platelets

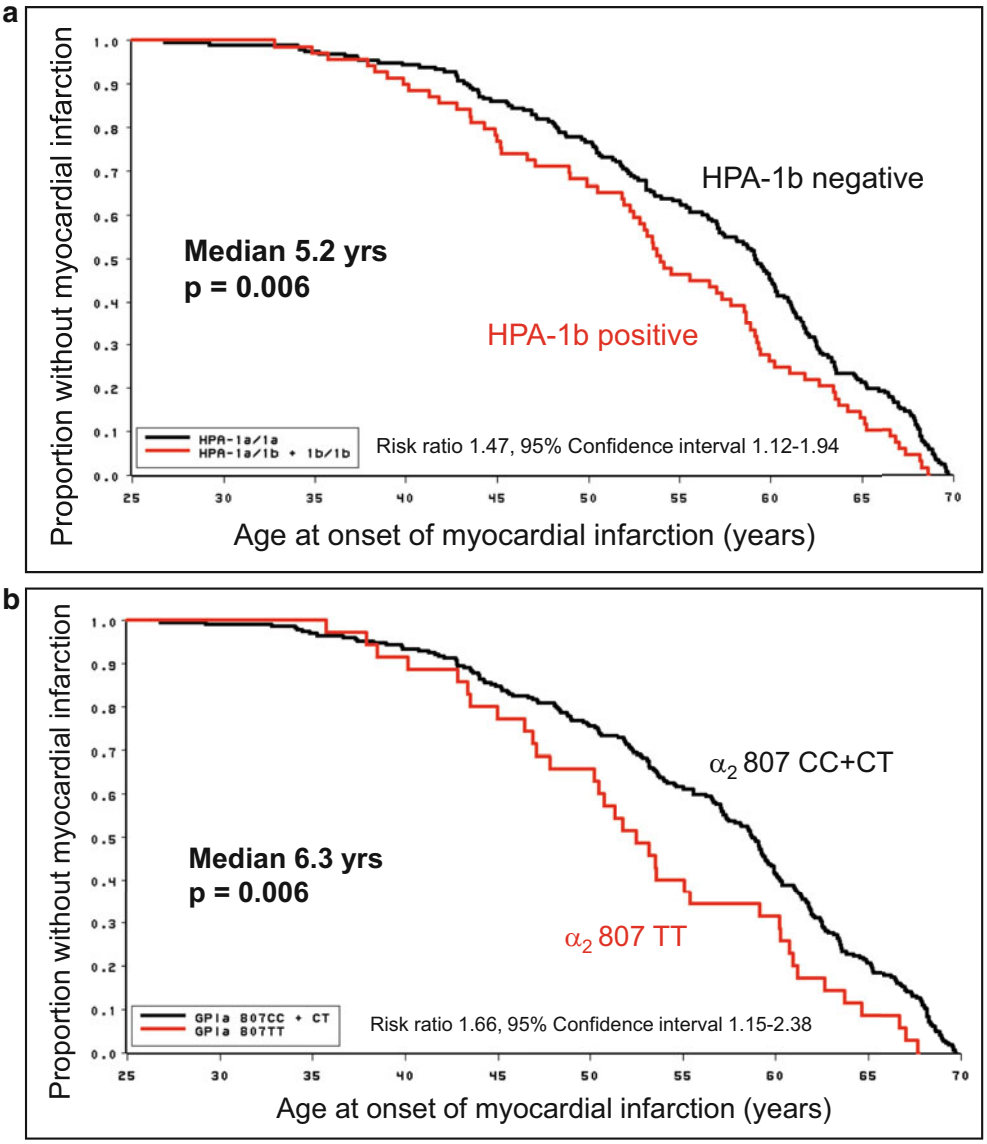


Fig. 3 Distribution of age at onset of myocardial infarction in relation to the HPA-1 (β3 C1565T) and α_2C807T polymorphisms of $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. The plots display the proportion of patients without myocardial infarction (MI) in the subgroup of patients with one- and two-vessel disease and recent-onset myocardial infarction recent (<1 year). The ages at onset of MI are referred to as event times, (**a**) in relation to HPA-1and (**b**) in relation to α_2C807T. The median age at onset of MI was 54.0 years in HPA-1b-positive patients and 59.2 years in the HPA-1b-negative patients ($p < 0.006$ by log-rank test). The median age at onset of MI was 52.5 in carriers of the $\alpha_2$807TT genotype and 58.8 years in carriers of the $\alpha_2$807CC or $\alpha_2$807CT genotype ($p < 0.006$ by log-rank test). Adapted from Zotz et al. (2005) and reprinted with permission of the publisher

is linked to the presence of arteriosclerotic vascular lesions (Zotz et al. 2005; Scharf and Zotz 2006).

Further aspects of platelet hyperreactivity in cardiovascular disorders are reviewed in Chap. 3.2.7. Increased platelet function associated with clonal hematopoietic proliferation will be discussed in the section on myeloproliferative neoplasms (Alimam & Harrison (2017) in this volume).

Decreased or Defective Platelet Function

Frequency of Acquired Platelet Disorders and Screening for Defects in Primary Hemostasis

Data on the rate of defects in primary hemostasis are available from a prospective analysis by Koscielny et al. (2004), who studied 5649 consecutive patients (age ranging from 17 to 87 years) prior to elective surgery. Aside from history and physical examination, the investigators used a bleeding questionnaire and a standardized test panel for hemostatic screening, including in vitro bleeding time determined by platelet function analyzer (PFA-100). Patients with preexisting hemostatic disorders or anticoagulation therapy were primarily excluded from the analysis. Of the 5649 patients, bleeding history was negative in 5021 (88.8 %); in the remaining group (11.2 %) with a bleeding history, screening for hemostatic defects was positive in 256 of the 628 patients (40.8 %). Diagnostic work-up of the 256 patients revealed platelet function defects in 73 %, coagulation disorders in 0.8 %, and combined hemostatic defects in 26.2 % (including a predominant proportion of patients with von Willebrand disease). Among the 187 individuals with defects in primary hemostasis, acquired platelet dysfunction was drug-induced in 162 patients (63.3 %); among them, 147 were taking aspirin, ticlopidine,

clopidogrel, or other nonsteroidal anti-inflammatory agents, while in 10 patients (6.2 %) antibiotic treatment appeared to be the cause of platelet dysfunction. It is conceivable that this rate of antibiotic-induced bleeding diathesis may be even higher in other patient groups. Indeed, as reported on a smaller cohort of hospitalized patients with prolonged Ivy bleeding time, 54 % were receiving large doses of antibiotics and 10 % were taking aspirin or other nonsteroidal anti-inflammatory agents (Wisloff and Godal 1981). The data by Koscielny et al. (2004) illustrate several important issues summarized in Box 3.

Drugs That Inhibit or Impair Platelet Function (See also other Chapters "Aspirin" Patrono (2017), "Inhibitors of $P2Y_{12}$" (Gross et al. 2017), "Glycoprotein IIb/IIIa Antagonists" (Shanmugasundaram and Moliterno 2017), "Dipyridamole and the PDE Inhibitors" (Gresele et al. 2017), "PAR-Antagonists" (Guimarães and Tricoci 2017) and "Inhibitors of Platelet Adhesion to VWF and Collagen" (Tersteeg et al. 2017)

Prototypes of antiplatelet agents and their mode of action are displayed in (Fig. 4). Acetylsalicylic acid (aspirin) and nonsteroidal anti-inflammatory agents, thienopyridines (clopidogrel, prasugrel, or ticagrelor), and integrin $\alpha_{IIb}\beta_3$ receptor antagonists (abciximab, eptifibatide, and tirofiban) are the most prominent causes of acquired platelet function disorders and, thus, of potential bleeding risk. Moreover, agents that increase intraplatelet cAMP, either by stimulation of its synthesis (prostaglandin E_1, prostacyclin) or by phosphodiesterase inhibition (dipyridamole, theophylline, caffeine), may also impair platelet function. Nitric oxide donors can increase the bleeding time and may contribute to hemorrhage, specifically in uremic patients. Antibiotics, particularly those of the β-lactam type such as penicillins and cephalosporins, may cause prolongation of the bleeding time even in normal volunteers. The mechanism, whereby these antibiotics impair platelet function, is currently not known, although it has been proposed that they bind to the platelet membrane and inhibit the interaction of agonists and von Willebrand factor (VWF) or modify platelet receptors chemically (George and Shattil 1991). Differences in antiplatelet effects (e.g., inhibition of aggregation) of carbenicillin, penicillin G, ticarcillin, ampicillin, nafcillin, azlocillin, piperacillin, alpacillin, or mezlocillin probably relate to differences in blood levels and drug potency. Adsorption and nonspecific binding to platelet membrane constituents are also discussed as mechanism, by which volume expanders can impair platelet aggregation; however, interference of dextran or hydroxyethyl starch with $\alpha_{IIb}\beta_3$ and its plasma ligands may be clinically relevant only in patients with a preexisting hemostatic defect.

Among psychotropic drugs, selective serotonin reuptake inhibitors (SSRIs) that are widely used for the treatment of anxious disorders and depression can cause abnormal bleeding (Halperin and Reber 2007). Specifically, agents with the highest degree of serotonin reuptake inhibition such as fluoxetine, paroxetine, and sertraline are associated more frequently with hemorrhagic events (Serebruany 2006). Various adverse effects implicating drug-induced platelet dysfunction have been reported upon the use of SSRIs. Abnormal bleeding time (Ivy) and prolongation of closure times (PFA-100) are frequent abnormalities upon screening together with platelet aggregation and secretion defects upon stimulation with ADP, collagen, epinephrine, or thrombin (Halperin and Reber 2007). The spectrum of antiplatelet effects induced by SSRIs is broad, including reduced platelet responsiveness, blockade of intraplatelet calcium mobilization, and lowered surface expression of GPIb-IX-V, integrin $\alpha_{IIb}\beta_3$, P-selectin, VLA-2, and PECAM-1 (Halperin and Reber 2007; Serebruany 2006).

In general, acquired platelet function defects secondary to drugs are mild and ubiquitous, considering, for example, the large number of individuals, who take aspirin regularly and therefore have impaired platelet function due to irreversible inhibition of cyclooxygenase-1 (COX-1)-dependent thromboxane A_2 (TXA_2) formation. The effect of aspirin ingestion on the hemostatic competency of normal volunteers has been debated but appears to be of minor relevance. Nevertheless, individuals taking aspirin chronically report a significant increase in bruising, epistaxis, and gastrointestinal blood loss. More importantly, there was a slight but statistically insignificant increase in hemorrhagic strokes in a group of otherwise healthy physicians who took aspirin chronically as primary prophylaxis against MI (Steering Committee of the Physicians' Health Study 1989). Besides aspirin, more than 250 pharmacological agents, foods (fish oils) or diets (eicosapentaenoic acid), spices (onion, garlic), and vitamins have been reported to impair platelet function (for review: Scharf 2012) (Box 4).

Taken together, for almost all agents interfering with platelet reactivity or inhibiting platelet function, data are limited to abnormal platelet aggregation studies in vitro or prolonged bleeding time, which do not necessarily reflect the true bleeding risk and, therefore, may have minor or questionable clinical importance. By contrast, treatment with antiplatelet agents can cause or aggravate severe hemorrhage in patients with preexisting hemostatic defects of any kind. This is particularly true for certain clinical settings (sepsis, disseminated intravascular coagulation), or conditions (cardiopulmonary bypass surgery, liver transplantation), and systemic or hematologic diseases that affect platelet function and can be associated with bleeding disorders (renal failure, liver cirrhosis, myeloproliferative

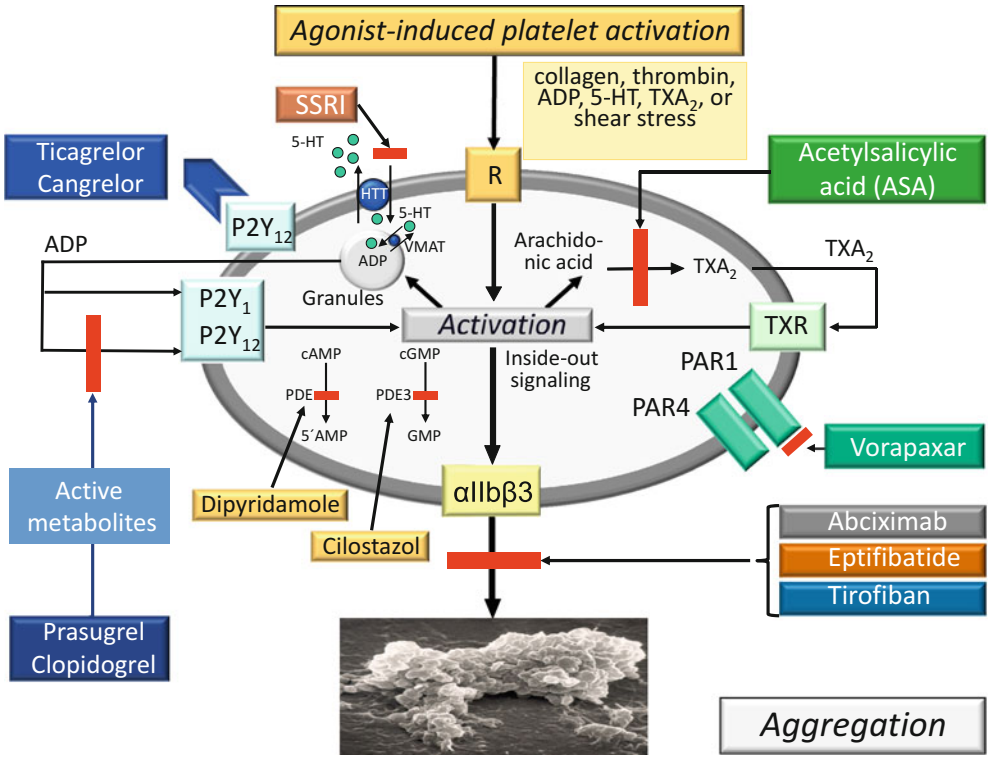


Fig. 4 Antiplatelet agents and their mode of action. Stimulation of circulating platelets with agonists such as collagen, thrombin, ADP, 5-HT, TXA_2, or by biomechanical forces such as shear stress results in platelet activation via inside-out signaling with conformational changes of integrin $\alpha_{IIb}\beta_3$ and subsequent platelet aggregation. Inhibition of TXA_2 formation or antagonism of the ADP receptor $P2Y_{12}$ interrupts positive feedback loops necessary for sustained platelet activation. Note that thienopyridines (clopidogrel and prasugrel) are prodrugs, which require conversion to their active metabolites, while the two newly designed direct and reversible platelet $P2Y_{12}$ antagonists, cangrelor and ticagrelor, are immediately active. Direct pharmacological blockade of $\alpha_{IIb}\beta_3$ by specific antibodies or receptor antagonists inhibits platelet aggregation. At approximately 15,000–20,000 intact residual $\alpha_{IIb}\beta_3$ receptor copies, antagonism is sufficient to suppress occlusive platelet thrombus formation in acute coronary interventions without inducing major bleeding. Abbreviations: ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; 5-HT, 5-hydroxytryptamine (serotonin); HTT, serotonin transporter; PAR, protease-activated receptor; PDE, phosphodiesterase; R, agonist receptor; SSRI, selective serotonin reuptake inhibitor; TXA_2, thromboxane A_2; TXR, thromboxane receptor; VMAT, vesicular monoamine transporter. Updated modification adapted from Scharf (2009) and reprinted with permission of the publisher

neoplasms, acute leukemias, myelodysplastic syndromes, and myeloma).

End-Stage Renal Disease (See also Lutz and Jurk 2017)

Bleeding may be a serious complication in patients with acute and chronic renal failure and is observed in up to one-third of uremic patients. Hemorrhages are also a concern in renal biopsy.

The pathogenesis of uremic bleeding is multifactorial (Table 1). It should be stressed that bleeding due to platelet abnormalities and other alterations involved in hemorrhagic complications is by far not the only hemostatic disorder in chronic renal failure. Thus, thrombosis of vascular access is common in patients undergoing hemodialysis. Moreover, uremic patients are highly susceptible to the development of atherosclerosis and its thrombotic complications (Boccardo et al. 2004).

Various platelet abnormalities, with partially conflicting results, have been reported in end-stage renal disease, including defects in platelet adhesion, aggregation, secretion, signal transduction, and metabolism of arachidonic acid and nitric oxide (Boccardo et al. 2004; Gawaz et al. 1994; Lutz et al. 2014; Oudemans-van Straaten 2015). More recently, it has also been shown that uremia can alter the platelet transcriptome (mRNA), thereby leading to an abnormal protein expression profile of platelets in patients with chronic kidney disease (Plé et al. 2012). The most consistent finding in end-stage renal disease is prolongation of the bleeding time, which may be abnormal in about 75 % of uremic patients. However, its value in predicting the risk of hemorrhage in uremia has never been

Table 1 Abnormalities and other determinants involved in the pathogenesis of bleeding associated with chronic renal failure. Data from Scharf and Schneider (1985), Scharf (1990), Gawaz et al. (1994), Boccardo et al. (2004), Plé et al. (2012)

Qualitative platelet abnormalities
– Reduced δ-granule content (ADP, serotonin)
– Reduced α-granule content (platelet factor 4, β-thromboglobulin) with
– Impaired secretion of α-granule constituents
– Elevated intracellular cAMP
– Abnormal mobilization of platelet Ca^{2+}
– Increased generation of nitric oxide by uremic platelets
– Defective cyclooxygenase activity
– Abnormal platelet arachidonic acid metabolism
– Abnormal platelet aggregation ex vivo in response to different stimuli
– Activation and ligand binding defects of $\alpha_{IIb}\beta_3$
– Altered platelet transcriptome (mRNA) with abnormal protein expression profile
– Uremic toxins, including urea, creatinine, phenol, phenolic acid, guanidine, guanidinosuccinic acid and parathyroid hormone
Quantitative platelet abnormalities
– Mild to moderate thrombocytopenia (due to inadequate production and/or consumption)
Abnormal platelet-vessel wall interactions
– Abnormal platelet adhesion
– Increased formation of vascular prostacyclin
– Abnormal production of nitric oxide
– Quantitative and/or qualitative alterations in plasma von Willebrand factor
Anemia
– Altered blood rheology
– Erythropoietin deficiency
Drugs[a]
– Antiplatelet agents
– Nonsteroidal anti-inflammatory drugs such as indomethacin, ibuprofen, and naproxen
– β-Lactam antibiotics
– Third-generation cephalosporins
– Selective serotonin reuptake inhibitors (SSRI) such as sertraline, paroxetine, and fluoxetine
– Heparin (during hemodialysis)

[a]Additional information can be obtained from Scharf (2012)

defined. Thus, numerous studies have failed to determine a consistent defect of platelet function or a clearly characterized abnormality in plasma VWF, both of which can be causally linked (Box 5).

Commonly, uremic patients with bleeding improve upon hemodialysis and transfusion of red blood cells. Nowadays, with appropriate continuous renal replacement therapy (CRRT) and the use of erythropoietin, the frequency and extent of bleeding episodes in renal disease have decreased (Oudemans-van Straaten 2015). In fact, reducing serum levels of uremic toxins that may cause or attribute to platelet dysfunction (Table 1), and increasing the hematocrit, thereby improving blood rheology, does correct several conditions, which otherwise contribute to defective hemostasis. Indeed, anemia is the main determinant of the prolonged bleeding time in uremic patients, as also demonstrated by an inverse relationship of increasing the hematocrit and concomitantly decreasing the bleeding time upon treatment with recombinant erythropoietin (Boccardo et al. 2004). However, the early termination of the Normal Hematocrit Cardiac Trial (Besarab et al. 1998), in which cardiac patients, who had been randomly assigned to the normal hematocrit group, presented with a higher mortality and a higher incidence of nonfatal MI, raised questions about the safety of the long-term maintenance of a normal hematocrit (Boccardo et al. 2004).

While it is generally assumed that hemodialysis corrects platelet abnormalities and reduces bleeding, it does not eliminate the hemorrhagic risk. Thus, platelet aggregation can be improved but also transiently impaired by CRRT, indicating that dialysis does not only remove inhibitors of platelet function but also causes activation and granular secretion upon platelet interaction with dialysis membranes (Scharf and Schneider 1985; Boccardo et al. 2004). For example, it has been demonstrated that hemodialysis induces a transient α-granule storage pool defect, leading to "exhausted" platelets that continue to circulate and exhibit defective TXA_2 formation (Stockschlader and Scharf 1989; Scharf 1990). This condition favors bleeding in uremic patients. Other platelet defects in patients with chronic renal failure are related to dysfunctional $\alpha_{IIb}\beta_3$ and defective interaction with fibrinogen or VWF (Escolar et al. 1990; Benigni et al. 1993; Gawaz et al. 1994). By contrast, binding of VWF to GPIbα and expression of GPIb-IX-V on platelets appear to be normal (Benigni et al. 1993). However, quantitative changes such as a decrease in total platelet GPIb content associated with an increase in soluble glycocalicin, a proteolytic GPIb fragment, have also been reported (Sloand et al. 1991).

Thrombocytopenia due to inadequate platelet production and/or consumption during regularly performed hemodialysis is also a common finding. However, platelet counts are rarely <80,000/μL and thus sufficient for primary hemostasis, presupposing intact platelet function. Other mechanisms that may cause a decrease in platelet counts are related to side effects of the anticoagulation regimen used for hemodialysis, in particular, to heparin-induced thrombocytopenia (Oudemans-van Straaten 2015).

Liver Disease (See also Lisman 2017)

Complex disorders of the hemostatic apparatus are present in acute and chronic liver disease, involving combined abnormalities of the megakaryocyte-platelet system, coagulation, and fibrinolysis. Apart from coagulation defects (INR >1.5) due to reduced hepatocellular synthetic capacity, thrombocytopenia of variable extent is a frequent feature both in acute and chronic liver disease. An analysis by the Acute Liver Failure Study Group, enrolling 1600 patients,

documented that the median platelet count on admission was approximately 130,000/μL; 60 % of patients had platelets counts <150,000/μL, 35 % <100,000/μL, and 10 % <50,000/μL (US Acute Liver Failure Study Group). In patients with advanced fibrosis or liver cirrhosis, the prevalence of thrombocytopenia ranges between 15 and 75 % (Giannini and Savarino 2008). The degree of thrombocytopenia appears to be proportionally related to the severity of liver disease but not associated with spontaneous bleeding, unless platelets counts decrease <50,000–60,000/μL (Scharf 1986a; Tripodi et al. 2006; Tripodi and Mannucci 2011).

Thrombocytopenia in liver disease results from increased lineal sequestration due to portal hypertension and subsequent hypersplenism (Scharf et al. 1983b; Scharf 1986a; Bashour et al. 2000), decreased thrombopoietin (TPO) production (Peck-Radosavljevic et al. 2000), and/or from toxic or virus-induced suppression of megakaryocytopoiesis (Scharf and Aul 1988; Bordin et al. 1995). In the past, low platelet counts in liver disease were mainly attributed to splenic pooling and/or consumption. More recently, the association between hepatic synthesis of TPO and residual hepatic function allowed a more specific insight into the pathophysiology of thrombocytopenia in liver disease. In fact, TPO levels are near-normal or even increased in acute hepatic failure (Schiodt et al. 2003). This is in contrast to patients with chronic liver disease, in whom TPO serum levels are significantly decreased and therefore thought to contribute to thrombocytopenia in cirrhosis (Giannini and Peck-Radosavljevic 2015). Treatment with TPO receptor agonists (e.g., eltrombopag) appears to be a promising therapeutic option to increase low platelet counts in patients with cirrhosis associated with hepatitis C (McHutchison et al. 2007). However, a double-blinded, randomized, placebo-controlled, phase 3 trial using eltrombopag prior to invasive procedures in patients with cirrhosis and thrombocytopenia was prematurely terminated because of an increased rate of thrombotic events (Afdhal et al. 2012).

Despite the multifaceted hemostatic defects, hemostasis-related bleeding episodes are relatively infrequent in patients with in liver disease. Based on clinical and systematic laboratory findings, we hypothesized as early as the mid-1980s that a balanced *low-level* hemostatic equilibrium due a *concordant* reduction in pro- and antihemostatic components is present in stable liver cirrhosis (Scharf et al. 1983b; Scharf 1986a). However, this balance is extremely labile and thus can be easily destabilized by various triggers (e.g., infection, variceal bleeding, decompensated liver cirrhosis, invasive procedures, or inadequate hemotherapy with prothrombotic components such fresh-frozen plasma, activated prothrombin complex concentrates, or recombinant factor VIIa). Recent studies have confirmed our contention of a *rebalanced* hemostasis resulting from a commensurate decline in pro- and antihemostatic factors both in patients with acute and chronic liver disease (Tripodi 2015; Lisman and Stravitz 2015). Moreover, despite decreased procoagulant coagulation factors, thrombocytopenia, and suspected platelet dysfunction, patients with acute or chronic liver disease can display hypercoagulable features, which may explain, at least in part, that thrombotic complications are more common than spontaneous bleeding complications (Tripodi 2015; Lisman and Stravitz 2015) (Box 6). For example, apart from normal or even enhanced thrombin generation in liver cirrhosis (Tripodi et al. 2005, 2011; Tripodi and Mannucci 2011), recent studies have shown increased levels of highly procoagulant platelet-derived microparticles (Stravitz et al. 2013). In addition, elevated plasma levels of VWF, typically observed in chronic liver disease, can compensate for the low numbers of circulating platelets (Lisman et al. 2006) and may restore primary hemostasis (Tripodi and Mannucci 2011; Tripodi 2015; Lisman and Stravitz 2015). However, these compensatory mechanisms fail to operate in patients with end-stage liver disease, in whom prominent bleeding complications remain a serious concern (Tripodi and Mannucci 2011).

Concomitant platelet function defects, as suggested in chronic liver disease, are less well defined. Generally, it has been assumed that platelet function deteriorates with the severity of liver failure. Older studies have described in vitro aggregation abnormalities in response to several agonists (Rubin et al. 1977; Weston et al. 1977). Some of the platelet aggregation defects were attributed to elevated levels of fibrinogen/fibrin degradation products or dysfibrinogenemia, both of which are rather common in chronic hepatitis and cirrhosis. Toxic effects of ethanol may also contribute to platelet dysfunction (Scharf and Aul 1988). Other possible mechanisms include defects in GPIb (Sanchez-Roig et al. 1994), decreased availability of arachidonic acid and consequently reduced TXA_2 formation (Laffi et al. 1988), increased cholesterol content of the platelet plasma membrane, impaired transmembrane signaling (Laffi et al. 1988), elevated sialic acid concentration of circulating platelets, and hypersialylated fibrinogen (Scharf 1986a). By contrast, antiplatelet antibodies, although rather frequently found in patients with acute or chronic viral liver disease, appear to be of minor relevance of qualitative or quantitative platelet disorders in this setting (Giannini and Savarino 2008). The diagnosis of impending DIC in patients with liver disease is often difficult to ascertain because of the multiple hemostatic alterations.

Disseminated Intravascular Coagulation (DIC)

Progressive thrombocytopenia and complex coagulation abnormalities are leading laboratory features in patients with DIC caused by a broad variety of conditions, including viral or bacterial infections and sepsis. Apart from pathogen-

induced thrombocytopenia, resulting from decreased platelet production, enhanced platelet consumption and clearance, or a combination of both, concomitant dysfunction of the circulating platelet population may aggravate bleeding at different stages of DIC. Some of the defects in primary hemostasis are attributed to "exhausted" platelets, an acquired storage pool deficiency that is believed to result from repetitive platelet stimulation in DIC (Pareti et al 1980; Scharf 1986a, b). Indeed, exhausted platelets continue to circulate (O'Brien 1978; Scharf 1990).

Generally, platelet dysfunction in DIC is not well characterized. For example, it has been suggested that elevated fibrin(ogen) degradation products, typically present in patients with DIC, are responsible for impaired platelet aggregation in vitro; however, the concentrations required to cause aggregation defects are unlikely to be found in vivo. The same is true for low fibrinogen levels (<100 mg/dL), which, except in cases with extreme hypofibrinogenemia, can rarely cause platelet aggregation defects, as platelet receptors are saturated at a fibrinogen concentration of only 10 mg/dL (Marguerie et al. 1984). More recently, sepsis-related alterations in platelet function have been studied in critically ill patients with and without DIC. Yaguchi et al. (2004) observed reduced in vitro platelet aggregability in septic patients, whereby the severity of sepsis was related to platelet aggregation defects. This observation can be interpreted in two ways: it may be that sepsis induces indeed a decreased platelet function, or, what is more likely that increased platelet aggregability is present in patients with sepsis that in turn will lead to circulating activated platelets, which will not respond properly in an ex vivo setting (Levi 2004). Likewise, Gawaz et al. (1997) found increased platelet surface expression of thrombospondin and platelet-neutrophil adhesion in septic patients. Overall, it appears likely that platelets become activated and hyperadhesive to neutrophils and endothelial cell in sepsis with and without DIC and that this mechanism promotes platelet sequestration, microcirculatory arrest, and in turn multiple organ failure.

Cardiopulmonary Bypass, Valvular Heart Disease, and Ventricular Assist Devices

Open-heart surgery using cardiopulmonary bypass (CPB) is accompanied by significant alterations, including "whole-body inflammatory response," reduction in coagulation factors, activation of fibrinolysis, and quantitative and qualitative platelet changes. Among these alterations, triggered by the contact between flowing blood and synthetic surfaces of the extracorporeal circuit, platelet dysfunction represents the major insult to hemostasis in this setting.

Thrombocytopenia secondary to CPB is caused by hemodilution and consumption due to adherence of platelets on the bypass circuit. CPB also results in shear stress-induced platelet activation, adherence onto immobilized adhesive plasma proteins, formation of platelet aggregates, secretion of granular constituents, platelet fragmentation, and generation of platelet-derived microparticles. Typically, CPB causes prolonged bleeding times, reduced platelet aggregation responses to several agonists in vitro, and acquired platelet storage pool deficiency, involving selective α-granule secretion or depletion of both α- and δ-granules (Bick 1985; Harker et al. 1980; Harker 1986; Kestin et al. 1993). Circulating degranulated ("exhausted") platelets due to activation induced by the interaction between blood and artificial surfaces were also identified in patients with chronic renal failure upon hemodialysis (Scharf and Schneider 1985; Scharf 1986a, b). Attempts have been made to prevent platelet activation during bypass and hemodialysis by infusion of prostaglandin E_1 or prostacyclin.

Highly abnormal rheologic conditions with excessive shear stress can also result in hemostatic defects with manifest bleeding. Acquired von Willebrand syndrome (aVWS) due to reduction or loss in large VWF multimers appears to be the underlying mechanism in suchlike settings. This has been convincingly shown in patients with severe aortic-valve stenosis (Vincentelli et al. 2003; Sadler 2003; Sucker et al. 2003) and subsequently also in patients with mitral regurgitation (Blackshear et al. 2014), in whom hemorrhagic complications due to aVWS resolved upon valve replacement. Interestingly, in aortic stenosis, the proportion of high-molecular-weight VWF multimers was inversely correlated with the mean transvalvular gradient (Vincentelli et al. 2003).

Acquired platelet dysfunction and qualitative and quantitative abnormalities in VWF are also frequently observed in patients with left ventricular assist devices (LVAD) and may cause otherwise unexplained bleeding complications (Steinlechner et al. 2009; Meyer et al. 2010; Baghai et al. 2015). The defects are attributed to abnormally high-shear conditions and the contact of circulating blood with artificial surfaces. Again, loss of high-molecular-weight VWF multimers appears to be the predominant pathomechanism in this setting; removal of the device is associated with complete restoration of the VWF monomer and multimer pattern (Meyer et al. 2010). Relevant pathologies of symptomatic defects in VWF are discussed in the Section on acquired von Willebrand syndrome.

Antiplatelet Antibodies

The overall impact of platelet-antibody interaction may be thrombocytopenia and platelet function abnormalities, as noted in the wide range of autoimmune disorders, including

idiopathic thrombocytopenic purpura (ITP), also designated immune thrombocytopenic purpura or immune thrombocytopenia (Ruggeri et al. 2008), or secondary causes such as systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid syndrome, viral infections (with cytomegalovirus, hepatitis C virus, human immunodeficiency virus, or varizella zoster virus), or side effects upon vaccination or administration of certain drugs (Neunert et al. 2011). Autoantibodies can induce platelet dysfunction by multiple mechanisms. In some patients, antibodies are directed against distinct platelet surface glycoproteins, including GPIa, GPIIa, GPIb, GPIIb, GPIIIa, GPIV, and GPVI.

In a traditional view, ITP was considered as being caused predominantly by antibody-mediated platelet destruction in the circulation at a rate that is not compensated by enhanced platelet production in the bone marrow. However, it has become evident that both megakaryocyte production and maturation and in turn thrombocytopoiesis can be also compromised by antiplatelet antibodies (McMillan and Nugent 2005; Bussel et al. 2006; Bromberg 2006; Nugent et al. 2009). Specifically, it was shown that TPO levels are inappropriately low for the degree of thrombocytopenia in the majority of patients with chronic ITP (Kuter and Gernsheimer 2009; Kuter 2009). This observation has provided a rationale for treatment with TPO mimetics in patients suffering from chronic refractory ITP (Bussel et al. 2006; Bromberg 2006; Kuter and Gernsheimer 2009; Kuter 2009; Neunert et al. 2011; Liebman and Pullarkat 2011). Two TPO receptor agonists, romiplostim and eltrombopag, have been approved for refractory postsplenectomy ITP patients. Currently, the use of these agents earlier in the disease course is debated but remains controversial (Liebman and Pullarkat 2011).

In the 1980s, two useful antigen-specific assays, the immunobead assay and the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay, were reported (McMillan et al. 1987; Kiefel et al. 1987). Applying these assays (specificities 78–93 %; sensitivities 49–66 %) (McMillan 2005), specific antiplatelet autoantibodies can be detected in 40–80 % of patients with clinical ITP (Liebman and Pullarkat 2011; McMillan 2009). Studying selected patients with chronic ITP, in about 60–70 % of cases, platelet-bound autoantibodies are directed against distinct epitopes on integrin $\alpha_{IIb}\beta_3$, while components of the (non-integrin) GPIb-IX-V complex are the antigenic target in approximately 5–20 % of cases (Liebman and Pullarkat 2011; McMillan 2009). In a subset of ITP patients, platelet-bound autoantibodies are directed concomitantly against $\alpha_{IIb}\beta_3$ and GPIb-IX-V or other platelet antigens. Less frequently, serum autoantibodies against platelets are also detected by either assay. More importantly, the absence of detectable antiplatelet autoantibodies (in about 20–30 % of patients) does not rule out the diagnosis of ITP. The reason for antibody-negative testing is unknown but may relate to heterogeneity of antigenic targets or methodological problems (Brighton et al. 1996).

Several patients have been described with autoantibodies directed against $\alpha_{IIb}\beta_3$ or GIb-IX-V, blocking the receptor-ligand interaction, thereby producing acquired Glanzmann thrombasthenia or acquired Bernard-Soulier syndrome (DiMinno et al. 1986; Niessner et al. 1986; Varon et al. 1992; Scharf 1996, 2003; Morath et al. 2005; Nurden and Nurden 2008). Inhibitory autoantibodies to GPIa and GPVI have also been found. Moreover, an ITP patient was identified, in whom binding of the anti-GPVI autoantibody resulted in the loss of GPVI from platelets (Boylan et al. 2004). Alloantibodies from Glanzmann thrombasthenia individuals with a history of frequent platelet transfusions can have the same thrombasthenia-like effect on transfused platelets (Coller et al. 1986).

Paraproteinemia and Amyloidosis

Qualitative platelet defects along with bleeding and symptomatic hyperviscosity are observed in approximately 30 % of patients with IgA myeloma or Waldenström macroglobulinemia (IgM), 15 % of patients with IgG myeloma, and occasionally in patients with monoclonal gammopathy of undetermined significance (Mehta and Singhal 2003). In the majority of cases with systemic amyloidosis, bleeding and platelet dysfunction appear to correlate closely with the plasma paraprotein concentration.

It has been suggested that some monoclonal immunoglobulins interact with the platelet surface and interfere nonspecifically with platelet adhesion or stimulus-response coupling. This contention has been supported by several observations summarized in Box 7.

Anecdotic cases of IgG myeloma have been described, where the paraprotein interacted with $\alpha_{IIb}\beta_3$ and blocked binding of fibrinogen, thus inducing a platelet defect of the Glanzmann thrombasthenia-like phenotype (DiMinno et al. 1986). In addition to platelet dysfunction and hyperviscosity syndrome, other causes of bleeding must be considered in myeloma patients, including thrombocytopenia, coagulation factor deficiencies by inhibitory paraproteins, impaired fibrin polymerization, and acquired von Willebrand syndrome (Bovill et al. 1986). Prolonged bleeding time and defective platelet adhesion due to interference of paraproteins with plasma VWF and the VWF-GPIb interaction have also been reported (Bovill et al. 1986; Mohri et al. 1987).

Similar hemostatic alterations are observed in amyloidosis (Sucker et al. 2006; Zangari et al. 2007). Prolongation of thrombin time, reptilase time, Russell viper venom time, prothrombin time, and activated partial thromboplastin time are found in most cases. Despite these frequent

abnormalities, major bleeding is present only in a minority of patients, who display associated hemostatic defects, including dysfunctional platelet aggregation, decreased coagulation factors (preferentially reduced factor X activity), abnormal fibrin polymerization, hyperfibrinolysis, and perivascular amyloid deposition (Sucker et al. 2006). Moreover, circulating paraproteins with associated amyloid and inhibitory effects on factor VIII and/or VWF have been reported (Zangari et al. 2007).

Acute Leukemias and Myelodysplastic Syndromes (MDS)

The most common cause of hemorrhages in these disorders is thrombocytopenia. In addition, acquired abnormalities of platelet function associated with clinical bleeding can be also present in acute myelogenous leukemia (AML) and, less frequently, in MDS. In the AML and MDS setting, qualitative and quantitative platelet changes result from abnormal megakaryo- and thrombocytopoiesis. Indicative of platelet dysfunction is a disproportionally prolonged bleeding time, or manifest bleeding, particularly from mucosal sites, at platelets counts >50,000/μL, which usually do not cause hemorrhages. Platelet abnormalities in AML and MDS include defective aggregation, storage pool deficiency, and reduced TXA_2 synthesis. Recently, it was reported in MDS patients that platelet aggregation defects result from dysfunctional $\alpha_{IIb}\beta_3$ and reduced expression of talin-1 (Fröbel et al. 2013).

Myeloproliferative Neoplasms (MNN)

Numerous structural, biochemical, and functional platelet changes have been described in patients with MPN (Table 2) (Schafer 1984, 2004; Wehmeier et al. 1989, 1990; Elliott and Tefferi 2004, 2005; Papadakis et al. 2010; Falanga and Marchetti 2014). Most likely, these heterogeneous alterations are the result of abnormal platelet populations derived from a malignant hematopoietic clone, but some of the abnormalities may be secondary due to enhanced platelet activation in vivo. The clinical impact of qualitative platelet defects observed in vitro is often unclear with widely divergent findings in different studies (Wehmeier et al. 1989, 1990, 1991a; Elliott and Tefferi 2005; Papadakis et al. 2010; Falanga and Marchetti 2014). Thus, platelet aggregation responses are highly variable and may vary in the same individual over time (Wehmeier et al. 1990). Likewise, platelet defects are demonstrable even in asymptomatic patients. Impaired responsiveness to epinephrine is a relatively frequent finding in MPN (>50 % of patients). The abnormality has been attributed to a decrease in the number of platelet α_2-adrenergic receptors (Schafer 1984).

Among the changes summarized in Table 2, functional defects of $\alpha_{IIb}\beta_3$ have been identified resembling Glanzmann variants. Thus, in flow cytometric studies, platelet aggregation abnormalities in MPN can be phenotypically characterized as (1) defects in $\alpha_{IIb}\beta_3$ activation, (2) defects in ligand binding, or (3) defects in postoccupancy events (Fig. 5). Other platelet alterations reported in MPN include abnormal arachidonic acid metabolism, defective signaling through the TXA_2 receptor, and impaired protein phosphorylation (Schafer 1984). Abnormalities in plasma VWF documented in patients with MPN are secondary and related to the degree of thrombocytosis, as discussed below.

Another unique feature of MPN is that thrombohemorrhagic events may occur concurrently, reflecting the complexity of pathomechanisms involved and also the difficulty of interpreting laboratory findings (Wehmeier et al. 1990; Schafer 2004; Elliott and Tefferi 2005; Papadakis et al. 2010).

Relevant Disease-Specific Somatic Mutations Significant advances in the molecular pathogenesis of MPN have improved our understanding of essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV) and may explain some of the overlapping clinical features in these settings. The abnormalities identified include somatic mutations in the tyrosine kinase Janus 2 gene *(JAK2)* and the thrombopoietin receptor gene *(MPL)*. The *JAK2* mutation results from a valine-to-phenylalanine (V617F) exchange and represents a gain-of-function variant, inducing increased phosphorylation of JAK2 substrates and increased responsiveness of myeloid cells (James et al. 2005). Approximately 50–60 % of patients with ET or PMF carry the *JAK2* V617F mutation that is also present in 95 % of patients with PV (Baxter et al. 2005). Activating mutations of the *MPL* gene are identified in about 5–10 % of patients with ET and PMF, who display nonmutated *JAK2* (Rumi et al. 2013). Subsequently, recurrent mutations in the gene encoding calreticulin (*CALR*) were detected in patients with ET or PMF but nonmutated *JAK2* or *MPL* (Klampfl et al. 2013; Nangalia et al. 2013). These molecular markers can fill the diagnostic gap that had existed in about 30–45 % of patients with ET or PMF so far. Very recently, novel noncanonical mutations (i.e., not V617F in *JAK2*) were reported in the minority of ET and PMF patients *without* established mutations in *JAK2*, *MPL*, or *CALR* genes ("triple-negative" MPN) (Milosevic Feenstra et al. 2016; Cabagnols et al. 2016). Interestingly, the presence of *CALR* mutations in ET appears to be associated with a better prognosis and a lower risk of thrombosis than in patients with the *JAK2* V617F mutation (Klampfl et al. 2013; Rotunno et al. 2014).

JAK2 V617F Mutation and Increased Platelet Function Several studies have explored the impact of disease-specific mutations on platelet function in MPN. When comparing platelets from *JAK2* V617F-positive with

Table 2 Morphologic, metabolic, and functional abnormalities in myeloproliferative neoplasms (MPN). Data from Wehmeier et al. (1990), Scharf et al. (1993)

Abnormalities	Specific findings
Heterogeneity in platelet size	
Morphologic abnormalities	Reduction in α- and δ-granules Alterations in the open canalicular and dense tubular systems Reduction in number and size of mitochondria
Acquired storage pool deficiencies	Decreased content of δ-granule constituents (ADP, ATP, serotonin)
Aggregation abnormalities	Highly variable; enhanced responses to agonists or even spontaneous platelet aggregation in some patients; diminished aggregation responses upon stimulation by ADP (40%), collagen (35%), or epinephrine (50–60%)[a]
Secretion	Highly variable; elevated plasma concentrations of α-granular constituents such as platelet factor 4 and β-thromboglobulin ex vivo, indicative of platelet activation; decreased secretion of δ-granule content (ADP, ATP, serotonin) caused by platelet storage pool deficiency
Platelet plasma membrane abnormalities	
– Glycoprotein receptor defects	Decreased expression of $\alpha_{IIb}\beta_3$, GPIb-IX-V, or $\alpha_2\beta_1$, increased expression of GPIV; dysfunctional $\alpha_{IIb}\beta_3$ with defective binding of soluble fibrinogen[b]; activation defects or other ligand binding defects of $\alpha_{IIb}\beta_3$; dysfunctional $\alpha_2\beta_1$
– Agonist receptor defects	Decreased number of α_2-adrenergic receptors; reduced responsiveness to TXA_2
– Alterations of Fc receptors	Increased binding of IgG
– PGD_2 receptors	Decreased number of PGD_2 binding sites
– Defective release of arachidonic acid	Reduced conversion of arachidonic acid to prostaglandin endoperoxides (TXA_2) or 12-lipoxygenase products (see metabolic abnormalities)
Metabolic abnormalities	
– Arachidonic acid metabolism	Enhanced synthesis of metabolites of the cyclooxygenase pathway (e.g., TXA_2); defective 12-lipoxygenase pathway with decreased formation of 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and 12-hydroxyeicosatetraenoic acid (12-HETE)
– cAMP and cGMP metabolism	Deficiency in cyclic adenylate cyclase and cyclic guanosine monophosphate (cGMP)-dependent protein kinase
Defective signaling	Abnormalities in calcium mobilization; reduced Ca^{2+} fluxes across platelet membranes; impaired protein phosphorylation (see cGMP metabolism)

[a]Rates in brackets indicate percentage of patients with defective aggregation responses (Schafer 1984)
[b]Enhanced binding of thrombospondin to the platelet surface of MPN patients has been observed in some but not in other studies (Wehmeier et al. 1991b)

JAK2 V617F-negative patients, an association between the *JAK2* V617F allele burden and increased platelet-mediated thrombin generation was documented (Panova-Noeva et al. 2011a). Other phenotypic features, as reported by Panova-Noeva et al. (2011b), are summarized in Box 8. Hobbs et al. (2013) studied a conditional *JAK2* V617F knock-in mouse model of ET, in which all megakaryocytes and platelets express *JAK2* V617F at a level, equivalent to that present in human ET patients. In this murine model, *JAK2* V617F-positive megakaryocytes displayed increased differentiation, higher proplatelet formation, and greater migratory ability. Concomitantly, platelet reactivity to different agonists was enhanced, as shown by a variety of assays (platelet aggregation and thrombus formation at arterial shear rates, spreading on fibrinogen, outside-in signaling, and platelet surface expression of activation markers). The increased platelet responses in vitro translated into increased hemostasis in vivo with markedly reduced duration of bleeding independently of the elevated platelet counts (Hobbs et al. 2013). These findings further suggest that *JAK2* V617F can induce intrinsic changes in both megakaryocyte and platelet biology beyond the effect on an increase in cell number.

Antiplatelet Therapy in MPN Aspirin is being widely administered for primary prophylaxis of vascular events in patients with ET and PV. However, the use of low-dose aspirin remains controversial in ET (Alberio 2016; Scharf 2016b). Several investigators recommend aspirin for *all* ET patients unless contraindicated (e.g., platelet count $>$ 1 mio/μL) (Beer et al. 2011; Cervantes 2011). Others restrict aspirin to high-risk patients (age $>$ 60 years, cardiovascular risk factors, previous thrombosis, *JAK2* V617F mutation), as assessed by risk scores (e.g., IPSET-thrombosis) (Barbui et al. 2012a; Passamonti et al. 2012), or to those with symptomatic microcirculatory disturbances (Schafer 2004; Harrison and Garcia 2014). Indeed, due to effective inhibition of enhanced platelet TXA_2 production, low-dose aspirin remains the treatment of choice in ET patients with erythromelalgia or transient neurological deficits.

While a significant benefit of low-dose aspirin has been convincingly documented for PV in the ECLAP study (Landolfi et al. 2004), no such prospective, double-blinded, placebo-controlled, randomized trial on aspirin exists in ET. Thus, the rationale for using aspirin in ET is not evidence-based but rather guided by analogies. Currently

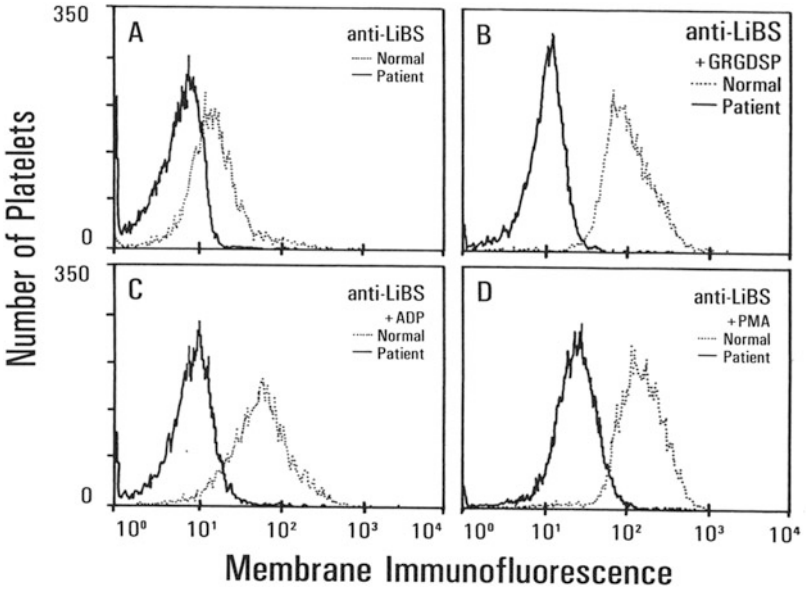


Fig. 5 Phenotypic characterization of dysfunctional integrin $\alpha_{IIb}\beta_3$ in myeloproliferative neoplasms associated with platelet aggregation defects. Depicted are flow cytometric histograms obtained from a patient with essential thrombocythemia (*solid line*) as compared with a normal subject (*dashed line*). Citrated whole blood was incubated with anti-LIBS-1, a conformation-specific monoclonal antibody that can distinguish between the resting, activated, and ligand-occupied state of $\alpha_{IIb}\beta_3$ in the absence (panel **a**) or presence (panels **b** through **d**) of other agents such as GRGDSP, adenosine diphosphate (ADP), or phorbol myristate acetate (PMA), an intracellular signal mimetic, which circumvents normal agonist receptor-mediated pathways by directly activating protein C kinase. Anti-LIBS-1 selectively reacts with a ligand-induced binding site on β3. This epitope is expressed upon receptor occupancy by fibrinogen (a process that requires platelet activation) or by fibrinogen-mimetic RGD-containing peptides (which bind to $\alpha_{IIb}\beta_3$ in an activation-*independent* manner). Immunolabeling of platelets with fluoresceinated anti-LIBS-1 in the presence of GRGDSP (panel **b**) or upon stimulation with ADP (panel **c**) resulted only in a minor increase of specific membrane immunofluorescence in the patient's platelets. Upon platelet activation with PMA (panel **d**), binding of anti-LIBS-1 increased but still differed significantly from that in a normal volunteer. These findings are indicative of a combined defect in activation and ligand binding of $\alpha_{IIb}\beta_3$ in the patient. The conclusion is supported by results obtained with PAC-1, a monoclonal antibody that specifically recognizes activated $\alpha_{IIb}\beta_3$ (data not shown). This diagnostic approach permits classification of platelet aggregation abnormalities as *(i)* defects of agonist-induced *activation* (no PAC-1 binding upon platelet stimulation by physiological agonists), *(ii)* defects of *ligand binding* (no LIBS expression and, consequently, no anti-LIBS-1 binding in the presence of fibrinogen-mimetic peptides), and *(iii)* defects of *postoccupancy processes*. The dysfunctional postoccupancy phenotype is characterized by defective platelet aggregation despite intact binding of PAC-1 and anti-LIBS-1 in response to platelet stimulation. Adapted from Scharf et al. (1993) and reprinted with permission of the publisher

available data in ET are limited and controversial. For example, in "low-risk" ET, an observational *case-control* study showed no difference in the incidence of thrombosis between untreated patients and healthy volunteers (Ruggeri et al. 1998), while a retrospective analysis suggested variable benefits for antiplatelet treatment (Alvarez-Larrán et al. 2010). Moreover, some studies included aspirin for all "high-risk" ET patients (Harrison et al. 2005), whereas other more recent studies did not (Gisslinger et al. 2013). These basic differences reflect a "dichotomy of opinion" in the field (Harrison and Garcia 2014). Essential concerns related to the use of antiplatelet agents in ET include drug-induced bleeding, specifically in ET patients with very high platelet counts, and "overtreatment" in patients whose disease is otherwise well controlled.

Another facet of thromboprophylaxis with aspirin is that its pharmacological inhibition of platelet COX-1 can be ineffective, in particular in high-risk ET patients, when low-dose aspirin (100 mg) is administered once daily. In fact, incomplete or absent suppression of TXA_2 production despite administration of aspirin results from accelerated renewal of platelet COX-1 due to enhanced clonal thrombocytopoiesis in ET (Pascale et al. 2012). Aspirin-insensitive TXA_2 biosynthesis can be reverted by modulating the aspirin-dosing interval (i.e., 100 mg twice daily) (Pascale et al. 2012).

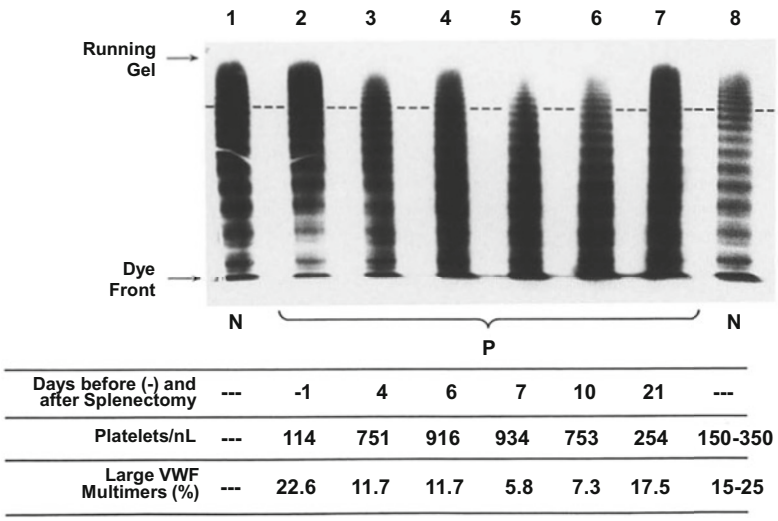
Altogether, the significance of antiplatelet treatment in ET using low-dose aspirin remains to be proven. Although aspirin appears to have more antithrombotic benefit than prohemorrhagic risk in this setting, drug-induced major bleeding remains a serious concern. Concomitantly, hemorrhagic complications in ET have been presumed to be due to acquired abnormalities in VWF. However, the proof of this association has not been demonstrated (Harrison and Garcia 2014). By contrast, it is evident that the abnormalities in VWF correlate with the degree of thrombocytosis, as discussed below.

Acquired Von Willebrand Syndrome (aVWS)

Bleeding events of the platelet-like phenotype can be caused by aVWS in a variety of conditions. A relevant pathomechanism of aVWS is increased proteolysis of intact VWF, which normally circulates as very large, homologous multimers composed of 250 kDa subunits (Sadler 2003). Upon exposure of VWF to high-fluid shear stress, present in valvular heart disease such as aortic-valve stenosis (Vincentelli et al. 2003; Sadler 2003; Sucker et al. 2003) and mitral regurgitation (Blackshear et al. 2014), or in cardiovascular assist devices (Steinlechner et al. 2009; Meyer et al. 2010; Baghai et al. 2015), circulating VWF multimers become most susceptible to cleavage by ADAMTS13, a plasma metalloproteinase that can be induced by high-shear conditions (Sadler 2003). Ultimately, its action leads to a deficit in large VWF multimers, which are particularly effective in hemostasis. Thus, shear stress-induced aVWS phenotypically resembles that of inherited mutations in VWF, which also increase its susceptibility to ADAMTS13 and cause von congenital Willebrand disease (VWD) type 2A. In both settings, a discrepancy between VWF: activity (ristocetin cofactor, RCo) and VWF: antigen (Ag) with discordantly lowered RCo can be present, resulting in an abnormally reduced VWF ratio (VWF: RCo: VWF: Ag, <0.7), indicative of a deficiency (or defect) in high-molecular-weight VWF species. However, this laboratory finding is only suggestive for VWD or aVWS and requires confirmation by multimeric analysis (Fig. 6).

The pathogenesis of aVWS can also result from decreased synthesis of VWF, as documented in association with hypothyroidism (Michiels et al. 2001), or from increased immune clearance of plasma VWF, as occasionally reported in patients with autoimmune, neoplastic, or lymphoproliferative disorders (Tefferi and Nichols 1997; Michiels et al. 2001). Various mechanisms may be involved, including inhibitory antibodies to VWF, formation of immune complexes between VWF and specific antibodies, and absorption of plasma VWF onto tumor cells (e.g., nephroblastoma), or abnormal lymphoid cells (e.g., in non-Hodgkin's lymphomas) (Michiels et al. 2001; van Genderen et al. 1994).

Myeloproliferative neoplasms, specifically ET at platelet counts exceeding 1 mln/μL, are rather frequently associated with aVWS. Affected patients have a decreased VWF: activity (RCo)/VWF: antigen ratio due to a decrease or absence of the largest plasma VWF multimers (Budde et al. 1984, 1986, 1993). A similar phenomenon is observed in patients with reactive thrombocytosis at excessive platelet counts, e.g., after splenectomy (Fig. 6). In clonal and reactive thrombocytosis, the underlying mechanisms appear to be the same: high-molecular-weight plasma VWF multimers are transiently bound to the increased mass of circulating platelets and preferentially degraded by ADAMTS13 (Shim



Days before (-) and after Splenectomy	---	-1	4	6	7	10	21	---
Platelets/nL	---	114	751	916	934	753	254	150-350
Large VWF Multimers (%)	---	22.6	11.7	11.7	5.8	7.3	17.5	15-25

Fig. 6 Multimeric structure of plasma VWF in a patient undergoing splenectomy because of chronic ITP. Blood samples were obtained before (lane 2) and after splenectomy (lanes 3 through 7), as indicated. VWF was identified with an anti-VWF antibody followed by immunoperoxidase staining. VWF multimers were detected by luminography and analyzed; their relative concentration was determined densitometrically. The high-molecular-weight VWF multimers are displayed at the top of the gel. The dotted line indicates the position of the tenth band resolved as an individual species; lager multimers were arbitrarily defined as those above the tenth band. N denotes normal control plasma (lanes 1 and 8). Note the normal distribution of multimers at a platelet count of 114,000/μL prior to splenectomy (lane 2); the progressive disappearance of the larger ones, as platelet counts increased following splenectomy (lanes 3 through 6); and the restoration of multimeric distribution upon normalization of the platelet count (lane 7). Taken from Budde et al. (1993) and reprinted with permission of The American Society of Hematology

et al. 2008). Thus, in both conditions, aVWS may explain the apparent paradox, why thrombocytosis can be associated with bleeding. Drug-induced aVWS has been described upon intake of pesticides or the use of valproic acid, certain antibiotics (ciprofloxacin, tetracycline) and mycotoxins (e.g., griseofulvin), thrombolytic agents, and hydroxyethyl starch (Michiels et al. 2001).

Conclusions and Future Directions

Acquired platelet disorders can be caused by a variety of clinical conditions and induce *increased* or *defective* platelet function. Precise identification of either phenotype, diagnosis of the underlying disease and knowledge of pathomechanism(s) resulting in abnormal platelet function, and appropriate translation into decision-making remain crucial in clinical practice (Box 9).

Acute vascular insults in arterial disease require primary intervention and secondary prophylaxis with antithrombotic, specifically antiplatelet agents. However, the expanding use of such agents has resulted in an increased proportion of the population being at risk for abnormal bleeding due to drug-induced platelet inhibition. This is especially true for elderly, comorbid patients. Hence, drugs represent the most common cause of platelet dysfunction in our overmedicated society. This also applies to a variety of pharmacological compounds, which by no means are aimed at interfering with platelet function (e.g., SSRIs for treatment of depression).

Regarding prevention and treatment of thromboembolic complications in patients with arterial disease, using antiplatelet agents (with or without combination of anticoagulants), we are currently facing two unresolved problems: firstly, the more intense antithrombotic therapy is, the more frequently major bleeding occurs; secondly, the agents used should be anti*thrombotic* but not anti*hemostatic*. In other words, we need effective drugs capable of preventing thrombus formation but not at the price of a concomitant risk of increased bleeding. This demand requires highly selective pharmacological strategies. Several innovative concepts have been designed and examined in animal models. For example, extensive efforts are being made to develop antiplatelet compounds that specifically block thrombotic effects without compromising primary hemostasis (Scharf 2016a). However, it remains to be seen whether these strategies can be really translated from mice to men. Thus, the novel agents under study warrant testing in preclinical and clinical trials.

Over the last decade, significant progress has been made to explore the nature of acquired hemostatic dysfunction in certain diseases. This applies primarily to hematologic disorders that can lead to *increased* or *decreased* platelet function and, along with these converse phenotypes, be associated with thrombotic or hemorrhagic complications. For example, characterization of malignant clones in MPN, specifically genotyping for mutations in genes encoding *JAK2*, *MPL*, and *CALR* in ET, PMF, or PV, is now being used in clinical risk scores to improve patient stratification and decision-making for or against antiplatelet treatment (Passamonti et al. 2012; Barbui et al. 2012a, b; Scharf 2016b). Moreover, recent insights into the kinetics of clonal thrombocytopoiesis in ET with enhanced platelet production that can circumvent COX-1 inhibition by low-dose aspirin, when administered once daily, have elucidated the phenomenon of incomplete or absent inhibition of platelet TXA_2 production (Pascale et al. 2012). Thus, it has been documented convincingly that the aspirin insensitivity in ET is due to accelerated renewal of platelet COX-1, the drug target, and that impaired platelet inhibition can be rescued by modulating the aspirin-dosing interval (i.e., 100 mg twice daily) rather than the dose (Pascale et al. 2012).

Hemostatic function and dysfunction in acute and chronic liver disease is another example for the significant progress that has been made in recent years. According to traditional paradigms, liver cirrhosis was considered as the epitome of an acquired coagulopathy that in combination with thrombocytopenia and/or thrombopathy causes hemorrhagic complications. Based on recent findings, this contention has been revised fundamentally in several aspects. Firstly, patients with liver failure are also prone to thrombotic events, which may be even more common than bleeding complications, except for end-stage liver disease. Secondly, the commensurate decrease in pro- and antihemostatic components can lead to a *rebalanced low-level* hemostatic equilibrium. The rebalanced hemostatic system is, however, labile and can be destabilized by various triggers, which in turn may explain the occurrence of both bleeding and thrombotic complications. Thirdly, thrombocytopenia in liver cirrhosis results from multifaceted causes; apart from lienal pooling, decreased production of TPO plays a major role, as shown by the correlation between TPO levels and residual hepatic function. Fourthly, defective platelet function as a concomitant cause of bleeding events in hepatic failure has been overestimated in the past. Fifthly, high levels of plasma VWF can restore platelet-vessel wall interaction and thus rebalance primary hemostasis that may be compromised otherwise in chronic hepatic disease. Sixthly, rebalanced hemostasis and, all the more, hypercoagulable features in liver disease have a major impact on the prevention and management of both bleeding and thrombosis. Thus, by contrast to common practice, a restrictive transfusion regimen is required in patients with liver disease to avoid prohemostatic hemotherapy and treatment-induced thrombotic complications. However, limitations of the progress made in this setting have also become evident in the meantime. For example, as a clinical consequence, eltrombopag, a TPO receptor agonists, was used in patients with cirrhosis and thrombocytopenia in order to increase platelet counts prior to elective invasive

procedures. Indeed, eltrombopag reduced the need for platelet transfusions but was associated with an increased incidence of thrombotic events (Afdhal et al. 2012). Consequently, early termination of the trial was mandatory.

Take-Home Messages

Box 1: Drugs represent the most common cause of acquired platelet dysfunction

Box 2: Platelet Receptor Polymorphisms

- Genetically determined integrin variants can modulate receptor expression and platelet function.
- Distinct integrin variants such as HPA-1b (Pro33) of $\alpha_{IIb}\beta_3$ and $\alpha_2$807TT of $\alpha_2\beta_1$ are associated with *premature* manifestation of myocardial infarction in patients with coronary artery disease.
- HPA-1b (Pro33) of $\alpha_{IIb}\beta_3$ is not a pro-atherogenic risk factor but a prothrombotic risk determinant that requires the presence of an atherosclerotic lesion to become effective, leading to increased platelet thrombogenicity.
- Indeed, HPA-1b (Pro33) of $\alpha_{IIb}\beta_3$ and $\alpha_2$807TT of $\alpha_2\beta_1$ have a prothrombotic phenotype in vitro.

Box 3: Frequency of Acquired Platelet Disorders and Screening for Defects in Primary Hemostasis

- Acquired platelet function defects are more frequent in clinical practice than generally believed.
- In the majority of cases, platelet dysfunction is drug-induced.
- Despite the broad use of antiplatelet agents, inhibitory effects of antibiotics should not be underestimated.
- Screening for defects in primary hemostasis requires appropriate laboratory techniques (e.g., PFA testing).
- Albeit trivial, the widespread practice of platelet counting and coagulation screening (APTT, prothrombin time) is inappropriate to identify individuals with platelet dysfunction.
- Pure coagulation defects occur less frequently than generally assumed in clinical practice.

Box 4: Drug-Induced and Disease-Associated Platelet Function Defects

- Acquired platelet dysfunction secondary to drugs is usually mild. By contrast:
- Treatment with antiplatelet drugs can aggravate hemorrhage in patients with preexisting hemostatic defects.
- Certain settings, conditions, and systemic or hematologic disorders may affect platelet function and cause bleeding:
  - Sepsis and disseminated intravascular coagulation.
  - Cardiopulmonary bypass surgery.
  - Liver cirrhosis and liver transplantation.
  - Renal failure and hemodialysis.
  - Myeloproliferative neoplasms, acute leukemias, myelodysplastic syndromes, and myeloma.

Box 5: Screening for Platelet Dysfunction in Renal Disease

- Prolongation of the bleeding time (BT) is a frequent finding in uremic patients.
- But BT is not a valid parameter in predicting the individual hemorrhagic risk in end-stage renal disease.

Box 6: Hemostatic Disorders in Liver Disease

- Coagulation defects (INR >1.5) in liver disease are not equivalent to pharmacological anticoagulation. By contrast:
- Patients with hepatic failure can display hypercoagulable features and are also prone to thrombotic events.

Box 7: Platelet Function Defects in Paraproteinemia and Amyloidosis

- Platelet dysfunction is more common at high paraprotein concentrations.
- Platelet aggregation, secretion, clot retraction, and platelet procoagulant activity may all be affected.
- Normal platelets acquire these defects upon incubation with patient plasma or purified paraprotein.

(continued)

Box 8: JAK2 V617F Mutation and Increased Platelet Function in MPN (Panova-Noeva et al. 2011a, b)

- Increase in platelet-mediated thrombin generation.
- Increase in immature platelets (which are hemostatically more active than mature platelets) in ET and PV.
- Linkage between amount of immature platelets and *JAK2* V617F mutation.
- Immature platelets in MPN are more susceptible to myelosuppressive treatment with hydroxyurea.

Box 9: Identification of Individual Patients with Preexisting Hemostatic Defects Remains Crucial

- To prevent otherwise unexpected bleeding complications
- To manage hemorrhagic symptoms adequately
- To minimize the risk from invasive procedures
- To avoid unnecessary exposure to blood products (including recombinant coagulation factor concentrates with adverse prothrombotic side effects)

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Part IV

Pathology: Thrombotic Disorders

Platelets in Arterial Thrombosis

Christian Schulz and Steffen Massberg

Abstract

Platelets play a key role in arterial thrombosis, a frequent and life-threatening condition. Upon vascular injury, platelets are rapidly recruited to the site of endothelial disruption, where they become activated and critically contribute to the formation of an occlusive clot. To treat or prevent arterial thrombosis, various antiplatelet therapies have been developed. However, many pathways targeted are also involved in hemostasis. Their inhibition therefore poses an increased risk of bleeding. To date it remains an eminent task to identify thrombosis-specific pathways in arterial thrombosis. Platelet receptors and intracellular molecules, whose absence does not cause overt bleeding in humans, may open up new therapeutic avenues. We here recapitulate the pathophysiology of arterial thrombosis and present newly identified pathways, which may serve as drug targets in the future. Finally, we will discuss the reciprocal interactions of platelets and innate immune cells and their potential role in arterial thrombosis. A detailed understanding of the mechanisms underlying this life-threatening condition is crucial for future research and the development of novel therapies.

Introduction

Arterial thrombosis is a major cause of mortality and morbidity worldwide. According to the recent Global Burden of Diseases Study 2013, ischemic heart diseases accounted for 7.0 million deaths worldwide (Collaborators 2015). Thus, there is an unmet clinical need to further improve patient treatment. While this requires a multimodal approach, antiplatelet therapies represent an important cornerstone of current treatment regimes. However, standard antithrombotic drugs that efficiently target thrombosis also affect hemostasis, which may offset the treatment benefit at the expense of bleeding. A deeper understanding of the processes underlying arterial thrombosis is therefore warranted to identify novel targets in thrombosis-specific pathways.

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Platelet Tethering and Firm Adhesion

The primary trigger for arterial thrombosis is the rupture of an atherosclerotic plaque. Under physiological conditions, platelets circulate in the bloodstream without significant binding to the vascular endothelium. Adhesion is prevented by endothelial nucleotide-hydrolyzing enzymes and mediators such as nitric oxide (NO) and prostaglandins, which reduce platelet activation and allow for an unperturbed blood flow (Marcus et al. 1997; Persson et al. 1990). Injury of the vessel wall exposes the thrombogenic subendothelial matrix (ECM) and initiates rapid platelet recruitment. The von Willebrand factor (vWF) receptor GPIb-V-IX axis plays a pivotal role in establishing platelet tethering to the vessel wall at arterial shear rates (Sakariassen et al. 1979; Savage et al. 1996) (Fig. 1). vWF derives mainly from the

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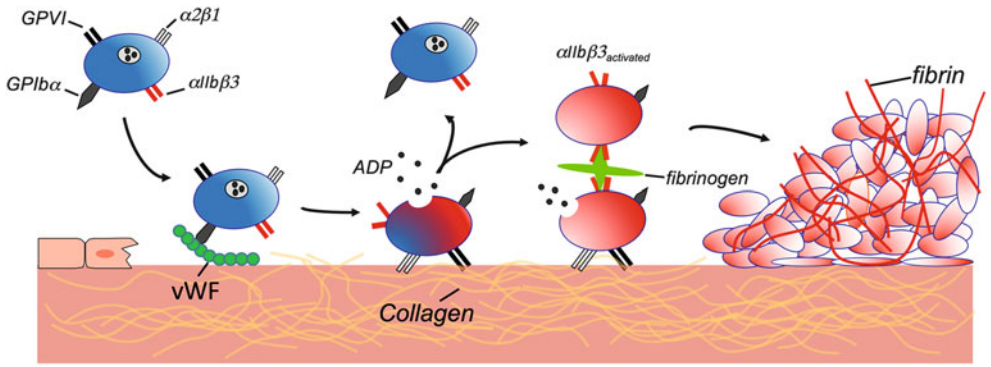

Fig. 1 Platelet adhesion in arterial thrombosis. The simplified schematic illustrates the principal steps of platelet recruitment and activation at the site of vascular injury. Initial tethering is mediated by GPIba interactions with surface-bound vWF resulting in slow surface translocation. The collagen receptors GPVI and integrin $\alpha_2\beta_1$ then mediate firm adhesion to fibrillar collagen exposed within the subendothelial matrix. Platelet activation leads to release of secondary mediators (e.g., ADP, TxA2) and $\alpha_{IIb}\beta_3$ activation, which culminates in platelet aggregation and clot formation (modified from Schulz and Massberg, Handb Exp Pharmacol 2012)

endothelium and is released upon injury toward both the luminal side and the subendothelial matrix. It is also synthesized by megakaryocytes and contained in platelets, which represent approximately 15 % of the circulating protein in blood (Howard et al. 1974). vWF immobilizes via its A3 domain to collagen fibrils (type I, III, and VI) within the ECM. Once exposed to high shear rates, conformational changes of vWF multimers allow the binding of its A1 domain to multiple platelet GPIbα receptors (Celikel et al. 2000). Platelet tethering via GPIbα-vWF interactions is characterized by rapid on-rates underscoring its importance at high shear rates which prevail in stenosed arteries. At very high shear stress, probably exceeding 400 dyn/cm^2, thrombus development is mediated solely by the interaction of GPIb with vWF and becomes independent of platelet activation (Ruggeri et al. 2006). Genetic ablation or antibody blockade of GPIbα abrogates arterial thrombosis (Bergmeier et al. 2006). However, the GPIb-V-IX receptor is also important for hemostasis. Consequently, patients with Bernard-Soulier syndrome as well as GPIbα-deficient mice develop a strong bleeding diathesis (Caen et al. 1976; Ware et al. 2000). Thus, complete ablation of GPIbα function does not represent a reasonable approach to treat or prevent arterial thrombosis. An alternative therapeutic strategy, which is currently under clinical investigation, is provided by specific inhibition of the GPIb-binding site on vWF (A1 domain). Because interactions of GPIbα and vWF become increasingly important at high shear stress, such approach may provide a more selective (shear-dependent) inhibition of platelet adhesion (Ulrichts et al. 2011). This concept is underscored by experimental models of thrombotic occlusion, in which increasing shear stress drives platelet binding to vWF-A1. This is specifically the case in boundary areas of newly formed thrombi that have not yet stabilized. In this setting, pharmacological blockade of GPIbα-vWF interactions has even been shown to disaggregate adherent platelets and restore patency of newly obstructed arteries (Le Behot et al. 2014; Momi et al. 2013). In contrast, stabilized fibrin-rich layers, which constitute inner parts of the thrombus, remain unchanged upon vWF-A1 antibody application. Thus, antithrombotic strategies targeting platelet-vessel wall interactions that occur predominantly at elevated shear rates might be promising to reduce platelet adhesion at specific pathologic conditions while having less impact on hemostatic plug formation.

While GPIbα is absolutely required for platelet adhesion at high shear rates, thrombus formation is reduced but not absent in vWF-null mice (Bergmeier et al. 2006). Thus, additional ligands exist which contribute to GPIbα-dependent platelet adhesion. Platelet-released thrombospondin-1 (TSP1) has recently been identified as an alternative adhesive ligand for GPIbα. It contributes to platelet adhesion at arterial shear stress (Jurk et al. 2003; Prakash et al. 2015) but also protects vWF from degradation by the cleaving protease ADAMTS13 and thereby fosters thrombus formation (Bonnefoy et al. 2006). While GPIbα is of paramount importance for initial binding to the injured vessel wall, the rapid off-rates of these bonds do not allow stable adhesion in the absence of other ligand-receptor interactions (Savage et al. 1998). Thus, GPIbα binding only reduces the velocity of tethered platelets thereby enabling interactions required for irreversible adhesion (Savage et al. 1996). Firm adhesive bonds are then formed by other platelet receptors, namely, integrins, which have relatively slow on-rates.

Integrins are heterodimeric proteins that consist of α- and β-subunits. Platelets express the β3-integrins $\alpha_{IIb}\beta_3$ (fibrinogen receptor) and αvβ3 (vitronectin receptor) and the β1-integrins $\alpha_2\beta_1$ (collagen receptor), α5β1 (fibronectin receptor) and α6β1 (laminin receptor). In nonactivated platelets, integrins are surface expressed in a resting conformation, which is shifted to a state of high affinity following

activation (inside-out signaling). For example, clustering of GPIbα during the initial adhesion step and consequent slow surface translocation represents an important activator of platelet integrins (Kasirer-Friede et al. 2002). Further, platelet-released factors, most prominently adenosine diphosphate (ADP) and thromboxane A2 (TxA2), induce and amplify platelet activation (Fig. 1). Likewise, ligation of platelet collagen receptors increases integrin affinity through inside-out signaling. This is noteworthy, because collagen represents the strongest activator of platelet integrins within the subendothelial matrix. Once activated, $\alpha_{IIb}\beta_3$ integrin binds to soluble fibrinogen resulting in firm platelet adhesion and aggregation (Shattil and Newman 2004). Exposure of the subendothelial matrix also allows binding of $\alpha_{IIb}\beta_3$ to collagen utilizing vWF as bridging molecule. Ligand binding to $\alpha_{IIb}\beta_3$ in turn triggers outside-in signaling, which activates various signaling complexes and maximizes platelet activation. Not surprisingly, genetic absence of the integrin domains α_{IIb} or β_3, the latter causing defects of both $\alpha_{IIb}\beta_3$ and αVβ3 integrins, abrogates arterial thrombosis in mice (Massberg et al. 2005; Smyth et al. 2001). Thus, $\alpha_{IIb}\beta_3$ inhibition is highly effective in arterial thrombosis and therapeutic antibodies for usage in human patients have consequently been developed (Gold et al. 1997). However, genetic absence of $\alpha_{IIb}\beta_3$ in humans causes Glanzmann thrombasthenia, which is characterized by significant bleeding diathesis, indicating that chronic inhibition of this integrin cannot represent an adequate strategy for continuous treatment or prevention of arterial thrombosis (Glanzmann 1951). And in the era of new $P2Y_{12}$ antagonists, even short-term inhibition of $\alpha_{IIb}\beta_3$ during coronary intervention is nowadays mostly restricted to usage as a bail-out strategy (Kastrati et al. 2004; Roffi et al. 2016). Specific targeting of activated platelets using conformation-specific antibodies could represent an alternative strategy to lower the bleeding risk of $\alpha_{IIb}\beta_3$ blockade (Schwarz et al. 2006). Novel approaches have focused on $\alpha_{IIb}\beta_3$-associated intracellular signaling pathways. Integrin-induced outside-in signaling depends on the G-protein-coupled receptor Gα13, which binds to its cytoplasmatic domain. Crosstalk of $\alpha_{IIb}\beta_3$ and Gα13 then promotes platelet spreading and clot retraction (Gong et al. 2010). These findings are in line with earlier observations that presence of Gα13 is critical for platelet thrombus formation and deficiency in Gα13 protects against arterial thrombosis in mice (Moers et al. 2003). However, primary hemostasis is also severely affected in these animals. In order to reduce negative effects caused by absence of Gα13, distinct signaling pathways have been addressed. Importantly, selective inhibition of the ExE binding motif of Gα13 blocks interactions with integrin β3 and selectively reduces outside-in signaling and platelet spreading, which is sufficient to abrogate arterial thrombosis. Because integrin ligation is not affected by the antibody, primary hemostasis is not altered (Shen et al. 2013). Thus, targeting of specific integrin-related pathways yields interesting therapeutic options in arterial thrombosis without causing a bleeding diathesis (Table 1).

In contrast to β3 integrins, platelet β1 can bind directly to collagen and other matrix molecules within the ECM. β1 integrins, however, seem dispensable for hemostasis. Even low levels (≥3 %) allow triggering of platelet signals required for normal hemostasis and bleeding time is not prolonged in β1 hypomorphic mice (Petzold et al. 2013). Nevertheless, near absence of β1 integrins significantly reduces arterial thrombosis in mice and therefore represents a potential therapeutic target for the treatment of arterial thrombosis without increasing the risk of bleeding (Petzold et al. 2013). A systematic comparison of platelet adhesive proteins recently indicated an important role of α6β1 in arterial thrombosis (de Witt et al. 2014). These findings have been corroborated in mice deficient in α6β1 integrin. Platelets from these animals are unable to adhere to the laminin isoforms composing blood vessels and consequently arterial thrombosis is markedly reduced (Schaff et al. 2013). Most importantly, hemostasis remains unaltered in this model, which suggests α6β1 as a suitable target for the treatment of arterial thrombosis (Table 1).

Platelet-Collagen Interactions

GPVI is the most prominent collagen receptor and plays a pivotal role in arterial thrombosis (Massberg et al. 2003; Nieswandt et al. 2001b). It represents a type I transmembrane glycoprotein and belongs to the immunoglobulin superfamily (Jandrot-Perrus et al. 2000). GPVI acts in concert with GPIbα to mediate platelet tethering at the site of vascular injury (Fig. 1); however firm adhesion can be achieved by direct binding of GPVI to the subendothelial collagen matrix (Nieswandt et al. 2001b). Not surprisingly, GPVI plays a pivotal role in arterial thrombosis following rupture of atherosclerotic plaque (Jamasbi et al. 2015). In line with the experimental observations in rodents described above, studies in human patients indicate that polymorphic variation at the GPVI locus is associated with an increased risk of myocardial infarction (Croft et al. 2001). Thus, GPVI could represent a potential target in arterial thrombosis.

Fibrillar collagen is the most prominent ligand of GPVI. Interestingly, various non-collagenous ligands have recently been identified suggesting that the role of GPVI in arterial thrombosis is not limited to its interactions with collagen. They include adiponectin (Riba et al. 2008), fibronectin (Bultmann et al. 2010; Maurer et al. 2015), and laminins (Inoue et al. 2006; Schaff et al. 2013), which can mediate platelet adhesion and activation via GPVI. Surprisingly, GPVI also binds to fibrin. This interaction amplifies platelet

Table 1 Role of selected platelet receptors and signaling molecules in arterial thrombosis and hemostasis in mice

Target	Mode of deletion/ inhibition	Injury model	Outcome	References
Platelet receptors				
β1 integrin	Hypomorphic β1 integrin mutation	Carotid artery ligation	Reduced platelet adhesion, normal hemostasis	Petzold et al. (2013)
	Genetic α2-integrin ($\alpha_2\beta_1$) deletion	Photochemical injury of the carotid artery	Reduced thrombosis, normal hemostasis	Chen et al. (2002), He et al. (2003)
	Genetic α6-integrin (α6β1) deletion	Mechanical injury of aorta, carotid artery, and mesenteric arterioles	Reduced thrombosis, normal hemostasis	Schaff et al. (2013)
β3 integrin	Genetic β3-integrin deletion	Clamp-induced carotid artery thrombosis and systemic intravascular thrombosis	Abrogation of thrombus formation, bleeding diathesis	Hodivala-Dilke et al. (1999), Smyth et al. (2001)
	Genetic αIIb-integrin ($\alpha_{IIb}\beta_3$) deletion	Carotid artery ligation and transient occlusion of the middle cerebral artery	Reduced arterial thrombosis and cerebral I/R injury, bleeding diathesis	Massberg et al. (2005)
GPIbα	Genetic deletion	$FeCl_3$ injury of mesenteric arterioles	Abrogation of thrombus formation, bleeding diathesis	Bergmeier et al. (2006), Ware et al. (2000)
GPVI	Genetic deletion	$FeCl_3$- or wire-induced injury of the carotid artery	Reduced thrombosis	Massberg et al. (2003)
	Antibody-induced receptor depletion	$FeCl_3$- or wire-induced injury of the carotid artery	Reduced thrombosis, moderate bleeding phenotype upon injury	Gruner et al. (2004), Massberg et al. (2003), Nieswandt et al. (2001a)
	Application of GPVI-Fc fusion protein	Carotid artery denudation and transient occlusion of the middle cerebral artery	Reduced thrombosis and cerebral I/R injury, moderate prolongation of tail bleeding times	Goebel et al. (2013), Massberg et al. (2004)
CLEC-2	Antibody-induced receptor depletion	$FeCl_3$ injury of mesenteric arterioles	Abrogation of thrombus formation, impaired hemostasis	Bender et al. (2013), May et al. (2009)
Fc receptor γ-chain	Genetic deletion	$FeCl_3$- or laser-induced injury of mesenteric arterioles	Reduced thrombosis, mild bleeding time prolongation	Kalia et al. (2008); Senis et al. (2009)
Gα13	Genetic deletion	Carotid artery ligation	Reduced thrombosis, bleeding diathesis	Moers et al. (2003)
	Inhibition of Gα13-integrin interactions	$FeCl_3$ injury of the carotid artery and laser injury of cremaster arterioles	Reduced thrombosis	Shen et al. (2013)
P2X1	Genetic deletion	Laser injury of mesenteric arterioles	Reduced thrombosis, normal hemostasis	Hechler et al. (2003)
P2Y1	Genetic deletion	ADP/collagen-induced pulmonary thromboembolism, $FeCl_3$ injury of mesenteric arterioles	Protection from thromboembolism, reduced arterial thrombosis, normal hemostasis	Fabre et al. (1999), Leon et al. (2003)
$P2Y_{12}$	Genetic deletion	$FeCl_3$ injury of mesenteric arterioles	Reduced thrombosis, prolonged tail bleeding time	Andre et al. (2003)
Intracellular signaling				
CalDAG-GEFI	Genetic deletion	$FeCl_3$ injury of mesenteric arterioles	Abrogation of thrombus formation, bleeding diathesis	Bergmeier et al. (2007)
	Deletion of the C1-like domain of CalDAG-GEFI	$FeCl_3$ injury of mesenteric arterioles	Reduced thrombosis, mild impairment of hemostasis	(Stolla et al. 2011)
CD148	Genetic deletion	$FeCl_3$- and laser-induced injury of mesenteric arterioles	Reduced thrombosis, impaired hemostasis	Senis et al. (2009)
DUSP3	Genetic deletion	$FeCl_3$ injury of the carotid artery	Abrogation of thrombus formation, normal hemostasis	Musumeci et al. (2015)
Orai1	Genetic deletion	$FeCl_3$ injury of mesenteric arterioles	Reduced thrombosis	Braun et al. (2009)
STIM1	Genetic deletion	$FeCl_3$ injury of mesenteric arterioles and mechanical injury of the abdominal aorta	Reduced thrombosis, mild bleeding phenotype	Varga-Szabo et al. (2008)

(continued)

Table 1 (continued)

Target	Mode of deletion/ inhibition	Injury model	Outcome	References
Platelet-released molecules				
MMP-2	Genetic deletion in bone marrow chimeric mice	Photochemical injury of the femoral artery	Moderate inhibition of thrombosis, mildly impaired hemostasis	Momi et al. (2009)
ERp57	PF4-dependent gene deletion	$FeCl_3$ injury of the carotid artery and of mesenteric arterioles	Reduced thrombosis, prolonged tail bleeding times	Wang et al. (2013)
PDI	PF4-dependent gene deletion	Laser injury of cremaster arterioles	Reduced thrombosis, normal hemostasis	Kim et al. (2013)

activation and procoagulant activity fostering thrombin formation and promoting platelet recruitment into the thrombus. In mouse arterial thrombosis models, GPVI-dependent binding to fibrin enhances thrombus growth and stabilization (Alshehri et al. 2015; Mammadova-Bach et al. 2015). Further, GPVI-activated platelets provide procoagulant activity by various means. For example, GPVI ligation induces phosphatidylserine exposure, which provides a platform for the binding of coagulation factors and thereby promotes thrombin and factor Xa formation (Munnix et al. 2005; van der Meijden et al. 2009). Further, GPVI-stimulated platelets release thiol isomerases, which directly activate $\alpha_{IIb}\beta_3$ integrin to foster platelet recruitment but also increase the activity of tissue factor, an important initiator of blood coagulation (Schulz et al. 2010; Wang et al. 2013). Because it has been unclear until recently how absence of GPVI not only protects from arterial thrombosis but directly reduces thrombus growth and stability (Bender et al. 2011), these findings close an important knowledge gap and link the function of GPVI in platelet activation and adhesion with a role in blood coagulation.

Importantly, absence of GPVI in humans and mice confers only a mild bleeding phenotype (Moroi et al. 1989; Nieswandt et al. 2000), supporting the suitability of GPVI as a therapeutic target in arterial thrombosis (Table 1). In addition to antibody blockade, GPVI function can also be inhibited by application of a soluble GPVI-FC dimer. In animal models of arterial thrombosis, GPVI-FC reduces thrombus formation and organ ischemia (Goebel et al. 2013; Massberg et al. 2004). The agent is currently undergoing clinical evaluation (ClinicalTrials.gov Identifier: NCT01645306) (Ungerer et al. 2011). Interestingly, chronic inhibition of GPVI not only inhibits platelet adhesion and protects from arterial thrombosis but also reduces atherosclerotic lesion formation in mice (Schulz et al. 2008). This is most likely due to inhibition of platelet activation and adhesion to collagenous structures within microerosions, which are eventually exposed at the atherosclerotic arterial wall. Further, collagen-GPVI interactions may induce platelet secretion (Ollivier et al. 2014), a process well known to be critical for the initiation of atherosclerotic lesion formation (Huo et al. 2003; Massberg et al. 2002). Thus, blockade of GPVI may not only prevent arterial thrombosis but could also impact on chronic inflammatory processes associated with atherogenesis and other conditions (Boilard et al. 2010).

The receptors involved in platelet adhesion and activation in arterial thrombosis depend on various factors, such as the prevailing shear stress as well as rheological conditions. It also depends on the composition of the subendothelial matrix. Collagen fibers and proteoglycans are main components of the ECM. They are embedded in elastin lamellae and together with smooth muscle cells form the tunica media (Wagenseil and Mecham 2009). Collagen types I and III represent the major component of the vascular matrix (Plenz et al. 2003) and account for up to 90 % of total collagenous protein in atherosclerotic lesions (Katsuda and Kaji 2003). In addition, also non-collagenic adhesion proteins such as fibronectin, vitronectin, and laminin are expressed (Watson 2009). However, the regional distribution of collagenous proteins varies strongly. Collagen type I accumulates preferably within the fibrous cap, whereas collagen type III is mostly found in the atheromatous core. Because platelets can adhere directly to collagen fibers via their collagen receptors, ECM composition is another factor determining the type of platelet receptors involved in adhesion. Indeed, $\alpha_2\beta_1$ integrin mediates adhesion preferably to collagen type III and thereby promotes shape change of aggregating platelets (Penz et al. 2005; Schulz et al. 2008). However, GPVI is significantly more important than $\alpha_2\beta_1$ integrin in platelet adhesion and aggregation induced by atheromatous plaque and represents the predominant collagen receptor in arterial thrombosis (de Witt et al. 2014; Nieswandt and Watson 2003).

In addition to sensing the composition of the adhesive matrix, e.g., by tethering to collagen fibers or surface-bound vWF, platelets seem to respond also to the matrix geometry. In fact, platelets are able to identify spatially restricted matrix cues during adhesion, which results in the directed release of their granule content toward this region. Platelet-derived matrix proteins (e.g., fibrinogen, fibronectin) and other molecules (e.g., P-selectin) then bind to the

subendothelial matrix (SEM) and extend the area available for platelet tethering and spreading (Kita et al. 2011; Sakurai et al. 2015). Whether this mechanism has a role in arterial thrombosis remains to be determined. However, it likely contributes to platelet adhesion at sites of minor vascular injury, such as plaque microerosions, and facilitates the recruitment of platelets to exposed SEM.

Platelet Activation and Intracellular Signaling

The detailed mechanisms of platelet activation and the underlying intracellular signaling cascades are described elsewhere in this book. However, recent findings have established a role of distinct signaling molecules in arterial thrombosis. We will therefore briefly highlight some of these pathways in the following paragraph.

Platelet function is controlled by intracellular calcium (Ca^{2+}) levels (Fig. 2). Thus, inhibition of signaling pathways responsible for changes in Ca^{2+} during platelet activation is likely to affect thrombus formation. Stimulation with various agonists culminates in activation of phospholipase C and production of diacylglycerol (DAG) and triphosphoinositol, which mediates rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR). Depletion of internal stores then triggers sustained Ca^{2+} influx through store-operated calcium entry (SOCE) channels located in the plasma membrane (Prakriya and Lewis 2015). In recent years the identity of some platelet Ca^{2+} channel regulators and their role in thrombosis have been revealed. Stromal interaction molecule 1 (STIM1) has been identified as an important Ca^{2+} sensor in the SR that controls Ca^{2+} release-activated channels (Luik et al. 2008; Park et al. 2009). Consequently, in STIM1-deficient mice, SOCE is absent and sustained Ca^{2+} influx is abrogated. This results in incomplete clot formation and protection of STIM1-knockout mice from arterial thrombosis and organ ischemia (Varga-Szabo et al. 2008). Hemostasis is only mildly affected probably because the rapid calcium burst from internal stores is sufficient in this context. Mechanistically, STIM1 oligomerizes and redistributes to the plasma membrane, where it associates with Orai1, the pore-forming subunit of Ca^{2+} release-activated channels, to trigger SOCE. Absence of Orai1 impairs Ca^{2+} entry, platelet secretion, and aggregation (Bergmeier et al. 2009; Braun et al. 2009). Together, STIM1 and Orai1 provide a crucial Ca^{2+} entry mechanism triggering platelet secretion and procoagulant activity. This pathway is mainly important for GPVI-mediated thrombus formation, while platelet activation through G-protein-coupled receptors (e.g., thrombin) is not altered (Gilio et al. 2010). In contrast to SOCE, receptor-operated calcium entry (ROCE) is mediated by ligand binding (e.g., ATP) to G-coupled receptors such as the cation channel P2X1. Transient Ca^{2+} influx via P2X1 receptors contributes to platelet activation and shape change (Rolf

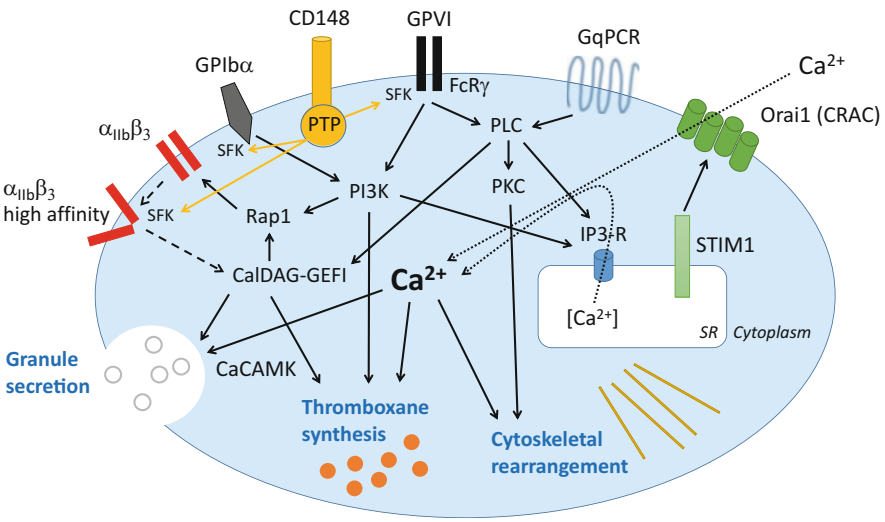

Fig. 2 Signaling cascades involved in platelet activation. The simplified model illustrates signaling molecules important in platelet biology. This includes the key stimulatory mediators PI3K, PKC, and PLC and the second messenger Ca(2+). They are critical for platelet activation, shape change, spreading, secretion, and integrin activation. Ca(2+) flux controls the activity of CalDAG-GEFI, which regulates platelet responses via Rap1 (modified from Bergmeier et al. Channels. 2013). The receptor-like PTP CD148 maintains Src family kinases (SFK) in an activated state which contributes to platelet activation downstream of various surface receptors. aIIbb3 integrin, fibrinogen receptor; GPIba, glycoprotein Ib-V-IX (vWF receptor); GPVI, glycoprotein VI (collagen receptor); GqPCR, Gaq protein-coupled receptor (e.g., thrombin receptor); Orai1, ORAI calcium release-activated calcium modulator 1; CRAC, Ca(2+) release-activated calcium channel; SFK, Src family kinases; PTP, protein tyrosine phosphatase; FcRg, Fc receptor common g chain; Rap1, small GTPase; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; CalDAG-GEFI, Ca(2+) and DAG-regulated guanine nucleotide exchange factor I; IP3-R, inositol trisphosphate receptor; STIM1, stromal interaction molecule 1; CaCAMK, Ca(2+)-calmodulin kinase; SR, sarcoplasmic reticulum

and Mahaut-Smith 2002). Interestingly, activation of P2X1 also amplifies Ca^{2+} signaling through P2Y1 and other Gq-coupled receptors (Jones et al. 2014). In the absence of P2X1, platelet aggregation and secretion in response to agonists are decreased, and in mice thrombus formation in arterioles and small arteries is inhibited (Hechler et al. 2003). However, the role of P2X1 in arterial thrombosis occurring in larger vessels remains elusive. In summary, calcium channels play a critical role in mediating platelet activation downstream of various agonists. Specifically, proteins regulating SOCE could represent interesting targets because of their imminent role in arterial thrombosis while causing only mild changes in the hemostatic response. However, due to their broad expression, genetic deficiency and gain-of-function mutations of the above-described proteins result in severe adverse effects on immune responses and tissue function (Lacruz and Feske 2015). Thus, further research is required to identify platelet-specific approaches for the inhibition of calcium channel regulators to reduce platelet activation in arterial thrombosis.

Small GTPases are cytosolic G-proteins, which regulate signal transduction processes involved in platelet granule secretion and integrin activation (Fig. 2). They are among the most abundant proteins in platelets (Burkhart et al. 2014). The guanine nucleotide exchange factor (GEF) calcium- and DAG-regulated GEF 1 (CalDAG-GEFI) is a critical regulator of small GTPases. It activates small GTPases, such as Rap1 and Rac1, and thereby activates β1 and β3 integrins in platelets (Bernardi et al. 2006; Crittenden et al. 2004). Absence of the CalDAG-GEFI encoding *RASGRP2* gene in humans causes a significant bleeding diathesis (Canault et al. 2014). However, presence of a single normal allele in heterozygous patients is sufficient to prevent bleeding while platelet adhesion and spreading on collagen remains absent. Thus, partial inhibition of CalDAG-GEFI may represent a safe therapeutic strategy in arterial thrombosis. In fact, in mice targeting of the regulatory C1 domain within CalDAG-GEFI reduces (but does not abrogate) its function and consequently decreases $\alpha_{IIb}\beta_3$ activation. Importantly, CalDAG-GEFI deficiency has only minor impact on hemostasis, less than inhibition of $P2Y_{12}$ (another pathway signaling through Rap1 activation) or genetic absence of Rap1 (Andre et al. 2003; Chrzanowska-Wodnicka et al. 2005). Thus, CalDAG-GEFI signaling represents a molecular pathway pivotal for platelet adhesion and thrombus formation, particularly under arterial shear conditions. However, its (partial) absence does allow the formation of hemostatic plugs, and therefore specific targeting of CalDAG-GEFI represents an interesting therapeutic strategy for the treatment of arterial thrombosis (Bergmeier et al. 2007; Stolla et al. 2011).

Finally, the signaling cascades induced by platelet-activating receptors, which mainly involve nonreceptor tyrosine kinases (NRTK) or G-protein-coupled receptors, may represent an interesting target for the inhibition of thrombosis. GPVI and the C-type lectin-like receptor 2 (CLEC-2) have been identified as major NRTK-coupled platelet-activating receptors both signaling through Src family kinases (SFK) and Syk (Lorenz et al. 2015; Severin et al. 2011). CLEC-2 deficiency abrogates platelet aggregation and arterial thrombosis in mice but also diminishes the hemostatic response (Bender et al. 2013; May et al. 2009). The pivotal role of tyrosine phosphorylation in the regulation of platelet function has been further corroborated by genetic deletion of regulatory molecules involved in downstream signaling pathways. For example, absence of the receptor-like protein tyrosine phosphatase CD148, a global activator of SFK signaling, abrogates thrombus formation and disrupts hemostasis (Fig. 2) (Mori et al. 2012; Senis et al. 2009). Syk tyrosine phosphorylation downstream of both CLEC-2 and GPVI signaling pathways is positively regulated by the dual-specificity phosphatase 3 (DUSP3). In mutant mice lacking DUSP3, platelet activation and arterial thrombosis are reduced (Musumeci et al. 2015). However, DUSP3 is dispensable for GPIbα binding and integrin $\alpha_{IIb}\beta_3$ outside-in signaling (mediating fibrin clot retraction) explaining why primary hemostasis is not affected in these animals. These findings nicely illustrate that selective inhibition of signaling pathways (e.g., downstream of GPVI/CLEC-2) yields interesting therapeutic options in arterial thrombosis, while ablation of regulatory molecules involved in a broad range of platelet functions is more likely to cause aberrant hemostasis.

Platelet-Released Molecules Modulating Arterial Thrombosis

Platelets contain a large repertoire of molecules, which are released upon activation and exert various functions in thrombosis and inflammatory conditions (Semple et al. 2011). Most of these molecules are actually platelet derived; however some factors are primarily circulating in blood, become internalized in platelets, and accumulate in their granule stores. ADP and TxA2 belong to the most established platelet mediators. They are released from activated platelets and act as secondary agonists to induce platelet integrin activation and aggregation (Offermanns 2006). However, recent studies have identified various other platelet-released mediators, which influence pathways involved in both platelet activation as well as blood coagulation and thereby impact on arterial thrombosis.

One example is plasma fibronectin (pFn). As outlined above, fibronectin is an important component of the ECM and represents a ligand for various platelet receptors, including $\alpha_{IIb}\beta_3$ integrin and GPVI. In contrast to cellular

fibronectin, which is generated and deposited locally, pFn is produced in the liver and circulates in blood. Interestingly, platelets internalize and accumulate pFn in larger amounts in a process involving $\alpha_{IIb}\beta_3$ (Ni et al. 2003a). In case of endothelial injury, pFn is readily deposited at the arterial wall and incorporated into the fibrin network, which is thereby strengthened. pFn promotes platelet aggregation and plays a role already in the initial phase of hemostasis (Wang et al. 2014). Consequently, depletion of pFN results in decreased thrombus formation and stability (Ni et al. 2003b), however, only in the presence of fibrin (Reheman et al. 2009). In the absence of fibrin, pFn inhibits platelet aggregation and thrombus formation (Wang et al. 2014). This mechanism seems to play a role in limiting platelet aggregation in the growing thrombus, particularly at border zones to the blood stream where fibrin is low or absent. By these means, pFn regulates thrombus growth and fine tunes the hemostatic response. However, in more severe vessel injuries, in which larger amounts of fibrin are generated (e.g., following plaque rupture), pFn switches its function to promote thrombus formation. Thus, pFn could represent a therapeutic target in arterial thrombosis, while its inhibition in the physiological process of hemostasis, in which it negatively regulates thrombus formation, probably is counterproductive.

In addition to proteins that interact with matrix components (e.g., fibrin) of a growing thrombus or the injured vessel wall, platelets also host molecules that directly promote fibrin formation and blood coagulation, i.e., proteins of the thioredoxin superfamily. They are generally contained in the endoplasmic reticulum (ER) of mammalian cells and play a central role in protein folding and processing of secretory proteins by enzymatic (e.g., disulfide bond formation) and nonenzymatic functions (Gething and Sambrook 1992). Interestingly, a growing body of work indicates a role in arterial thrombosis. Various thiol isomerases have been identified in platelets including ERp5, ERp57, and protein disulfide isomerase (PDI). Upon activation, they are mobilized to the cell surface and secreted; however, they can also bind to $\alpha_{IIb}\beta_3$ and thereby remain surface bound (Holbrook et al. 2010; Schulz et al. 2010; Wang et al. 2013). In fact, $\alpha_{IIb}\beta_3$ is a major target of these proteins, and direct interaction with the β3 integrin during its activation is required for incorporation of platelets into a growing thrombus (Cho et al. 2012; Passam et al. 2015; Wang et al. 2013). Besides inducing platelet activation and aggregation, thiol isomerases are also critical effectors of blood coagulation. PDI and ERp57 have been shown to trigger conversion of tissue factor (TF) from the functionally inactive to the active form (Reinhardt et al. 2008; Schulz et al. 2010). This process is referred to as TF decryption and represents an initial step in coagulation activation. Thus, platelet-released thiol isomerases support TF-dependent blood coagulation at sites of vessel damage in mice (Kim et al. 2013; Reinhardt et al. 2008). Notably, the endothelium represents another source of these proteins, and the precise contribution of endothelium versus platelet derived thiol isomerases in arterial thrombosis is subject to ongoing investigations. It not only seems to vary for the individual proteins but also depend on the mouse injury model analyzed (Jasuja et al. 2010; Passam et al. 2015). Further, thioredoxin family members are close structural homologues and recognize common substrates; however they may also bind to distinct domains and have specialized functions (Maattanen et al. 2006). This observation has been corroborated in the context of platelet biology. For example, the absence of platelet ERp57 prolongs tail bleeding times, whereas hemostasis is not altered in mice with PDI-deficient platelets (Kim et al. 2013; Wang et al. 2013) (Table 1). In line with this, combined antibody blockade of ERp5 and PDI has additive but not synergistic effects on platelet aggregation (Jordan et al. 2005). Thus, the precise role of individual thiol isomerases in arterial thrombosis awaits further experimental data. Nonetheless, genetic deficiency as well as antibody blockade of either ERp5, ERp57, or PDI reduces platelet aggregation and thrombus formation in mice in vivo (Kim et al. 2013; Passam et al. 2015; Reinhardt et al. 2008; Wu et al. 2012; Zhou et al. 2014). Thus, inhibition of these proteins represents a potential strategy in arterial thrombosis. Particularly PDI, whose absence is not associated with overt bleeding in animal models, seems to be a promising drug target (Jasuja et al. 2012). A first clinical trial addressing PDI inhibition in patients with cancer-associated thrombosis is currently under way (ClinicalTrials.gov Identifier: NCT02195232).

Various other proteins exist in platelets of which we have only begun to understand their functions and potential implications in arterial thrombosis. They include Gas6, a protein encoded by the growth arrest-specific 6 gene, which has previously been shown to play a role in inflammatory processes involving leukocytes and endothelial cells. Platelets are an important source of Gas6 but also contain receptor tyrosine kinases on their surface that bind Gas6 as a ligand. Gas6 binding then induces platelet secretion, $\alpha_{IIb}\beta_3$-dependent outside-in signaling, and aggregation (Angelillo-Scherrer et al. 2005; Gould et al. 2005). Importantly, the absence of Gas6 is associated with thrombus instability and protects mice from arterial thrombosis. Similar results have been observed in the absence of either of the three known platelet Gas6 receptors (Angelillo-Scherrer et al. 2001, 2005; Cosemans et al. 2010; Gould et al. 2005). Future studies will have to define in more detail the suitability of Gas6 as a therapeutic target in arterial thrombosis.

Matrix metalloproteinases (MMPs) play an important role in tissue remodeling and contribute to vascular inflammation. Their identification in platelets has opened another chapter in platelet activation and thrombosis. Platelets

contain MMP-1 (not in *Mus musculus*), MMP-2, MMP-3, and MMP-14 (also known as membrane type 1 [MT1]-MMP), all of which can be secreted upon activation (Kazes et al. 2000; Sawicki et al. 1997). MMP-1 and MMP-2 have been shown to promote platelet activation and aggregation in vitro (Fernandez-Patron et al. 1999; Galt et al. 2002), and MMP-2 enhances arterial thrombosis in mice in vivo (Momi et al. 2009). MMPs also modify the extracellular matrix by exerting lytic activity and degrading collagen fibers. This potentially limits thrombus formation at later stages of arterial thrombosis when platelet MMP activity and secretion reaches a maximum (Mastenbroek et al. 2015). Thus, platelet-released MMPs can be considered a potential therapeutic target in arterial thrombosis (Table 1). This is specifically interesting as conventional antiplatelet agents, namely, acetylsalicylic acid, do not prevent the release of MMP-2 from activated platelets (Falcinelli et al. 2007).

Because platelets host numerous proteins with agonistic and pro-inflammatory functions (Semple et al. 2011), it is interesting to note that some molecules act as negative regulators of platelet activation. They include carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1), endothelial cell-specific adhesion molecule (ESAM), junctional adhesion molecule-A (JAM-A), and platelet endothelial cell adhesion molecule-1 (PECAM-1). Consequently, absence of these molecules results in gain of platelet function and susceptibility to thrombosis. The mechanisms by which these mediators inhibit platelet activation are incompletely understood. CEACAM1 and PECAM-1 seem to act predominantly through reducing collagen-dependent platelet activation (Falati et al. 2006; Jones et al. 2001; Wong et al. 2009), while JAM-A acts as an endogenous inhibitor of integrin outside-in signaling (Naik et al. 2014, 2012). The precise function of ESAM remains to be explored. The protein has been shown to cluster in areas of homotypic platelet interactions and might therefore interfere with contact-dependent platelet activation. Absence of ESAM in mice confers increased platelet integrin activation and arterial thrombosis (Stalker et al. 2009). Together, regulatory proteins that inhibit platelet functions may deem useful in arterial thrombosis. It should be noted, however, that platelets are not the only source of these molecules and that endothelium and other blood cells may also produce and secrete them.

In summary, platelet-derived molecules may affect arterial thrombosis in multiple ways. They may interact with fibrin and matrix proteins at the vessel wall (e.g., pFn), promote blood coagulation (e.g., TF activation by thiol isomerases), and directly promote (e.g., ADP, Gas6, TxA2) or inhibit (e.g., CEACAM1, ESAM, JAM-A) platelet activation. The plethora of molecules that is potentially released from activated platelets and modulates their function is intriguing. Future research will have to define in more detail the specific pathological conditions, in which certain factors are of critical importance, their mechanisms of action, and whether they can be harnessed for the development of novel therapeutic approaches.

Platelet Interactions with Innate Immune Cells

Circulating innate immune cells act as a central line of defense against invading pathogens. Besides providing an array of bactericidal mechanisms, they promote pro-coagulatory pathways to initiate thrombus formation within the microvasculature, which contributes to the containment and elimination of pathogens. Thus, thrombosis represents an important mechanism of intravascular immunity (Engelmann and Massberg 2013). Interestingly, innate immune cells also represent a common cellular substrate in human arterial thrombus specimen (Mangold et al. 2015; Riegger et al. 2016) (Fig. 3a, b). The pathophysiology and clinical consequences are incompletely understood; however, reciprocal activation of platelets and immune cells likely contributes to arterial thrombosis. Platelets can directly recruit leukocytes to sites of injury, and in the absence of circulating platelets, recruitment of myeloid cells is strongly reduced (Schulz et al. 2011). Activated platelets express a broad spectrum of adhesion molecules on their surface, which allows interactions not only with the vessel wall but also with circulating blood cells (Fig. 3a). The most prominent ligand is P-selectin, which is expressed on activated platelets and mediates aggregate formation with various leukocyte subsets, mostly monocytes and polymorphonuclear neutrophils (PMN). In patients with acute coronary syndrome, platelet-leukocyte-aggregate (PLA) formation is increased and coincides with the early phase of arterial thrombosis (Furman et al. 2001; Sarma et al. 2002). Platelets also form aggregates with lymphoid cells under flow; however, the pathophysiological relevance of this interaction remains to be elucidated (Diacovo et al. 1998; Marquardt et al. 2009). Aggregation of platelets and myeloid cells is mostly established via binding of P-selectin to P-selectin glycoprotein ligand-1 (PSGL-1) (Yokoyama et al. 2005). However, blockade of P-selectin on activated platelets only partly reduces leukocyte adhesion under arterial flow conditions, clearly indicating the involvement of additional ligand-receptor pairs. Alternatively, interactions of the membrane proteins CD154 (CD40 ligand) and CD40 mediate PLA formation. Further, platelet GPIbα and αbβ3 integrins bind to leukocyte β2-integrins (CD11b, MAC-1) and cellular adhesion molecules (e.g., ICAM-1), respectively, whereas platelet GPVI has been shown to bind CD147 on myeloid cells (Diacovo et al. 1996; Schulz et al. 2011; Seizer et al. 2009).

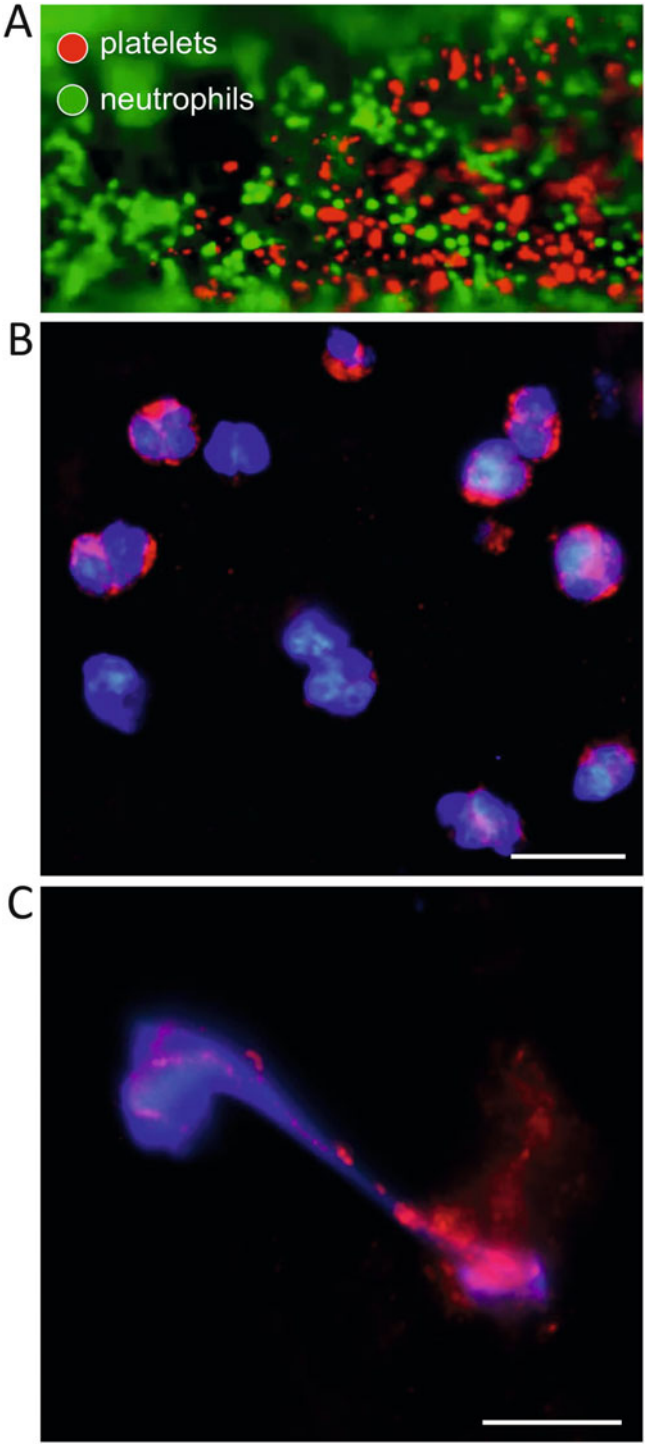

Fig. 3 Immune cells in arterial thrombosis. (**a**) Intravital microscopy of arterial thrombosis in a mouse carotid artery injury model. Platelets (*red*) and neutrophils (*green*) closely interact during thrombus formation at the injured vessel wall. (**b** and **c**) Leukocyte accumulation in thrombus specimens retrieved from coronary arteries of patients with myocardial infarction (Riegger et al. 2016). (**b**) Immunofluorescence staining of a coronary thrombus identifies neutrophils (*red*, neutrophil elastase (NE) staining) and nuclei (*blue*, Hoechst). Bar, 10 mm. (**c**) Activated neutrophils release prothrombotic nucleosomes (*blue*) in a process termed NETosis. Bar, 5 mm

Activated platelets foster prothrombotic pathways in myeloid cells (Celi et al. 1994; Lindmark et al. 2000; Sako et al. 1993). Among others, this leads to formation of TF by monocytes, which culminates in fibrin deposition and thrombus stabilization (Palabrica et al. 1992). In PMN, platelet-secreted molecules such as P-selectin and high-mobility group box 1 induce neutrophil activation and NET formation (Etulain et al. 2015; Maugeri et al. 2014). NETs are scaffolds of extracellular nucleic acids that act as strong procoagulant surfaces and propagate local coagulation (Engelmann and Massberg 2013; Martinod and Wagner 2014) (Fig. 3c). They can bind to and activate platelets and other procoagulant effectors such as factor XII (Brinkmann et al. 2004; Fuchs et al. 2010; von Bruhl et al. 2012). NETs also bind to TF, which is activated and then triggers the extrinsic pathway of blood coagulation (Reinhardt et al. 2008). Thus, NETs provide several mechanisms to foster fibrin formation and potentiate thrombus stability but also directly induce platelet activation and aggregation, suggesting that inhibition of NET formation represents an interesting therapeutic strategy in arterial thrombosis. This is corroborated by recent data in patients with myocardial infarction showing that the number of NETs in coronary thrombi correlates positively with infarct size (Mangold et al. 2015). Together, reciprocal activation of platelets and myeloid cells, together with fibrin formation and the induction of pro-coagulative pathways, plays a decisive role in arterial thrombosis. Thus, the inhibition of immune cell activation and recruitment may open up new therapeutic avenues for the treatment and prevention of this condition. Further, effective platelet inhibition is likely to reduce the number of leukocytes recruited into the growing thrombus and may also abrogate their sequelae (e.g., NET formation).

As a side note, platelets not only directly recruit leukocytes to sites of injury (via ligand-receptor interactions) but also indirectly through release of soluble factors. These include chemotactic mediators, such as CCL5 (regulated on activation normal T cell expressed and secreted, RANTES), CXCL4 (platelet factor 4, PF4), or stromal cell-derived factor 1α (SDF-1α), which are abundant in platelets. Once released upon activation, they recruit and bind leukocytes and hematopoietic progenitor cells at the site of injury. However, instead of acute thrombosis, this mechanism is probably more important in chronic pathologies such as atherogenesis or post-injury remodeling (Massberg et al. 2006; Schober et al. 2002).

In summary, inhibition of platelet-immune cell interactions as well as their bidirectional activation paths is likely to represent a novel therapeutic option in arterial thrombosis. Specifically P-selectin represents an interesting target because of its leading role in PLA formation (Michelson et al. 2001). Consequently, antibodies for in human use

have been developed. In a first placebo-controlled randomized trial in patients with acute coronary syndrome, P-selectin blockade abrogated PLA formation and neutrophil activation. The antibody-treated group also showed a trend toward reduced myocardial injury (Tardif et al. 2013). Further studies will have to decipher the consequences and benefit of P-selectin blockade in humans.

Outlook

Arterial thrombosis is an acute and life-threatening condition with severe impact on morbidity and mortality worldwide. Despite improvements in antithrombotic therapies, there is an unmet clinical need in strategies protecting from thrombosis while leaving physiological hemostasis unaffected. Ongoing and future research will have to unravel the mechanisms and molecular cues specific for thrombosis-related pathways. GPVI and integrin α6β1 represent examples of platelet receptors, whose absence does not cause overt bleeding. Likewise, interference with crucial signaling pathways by targeting distinct binding motifs (e.g., Gα13-EXE, vWF A1, or CalDAG-GEFI C1 domain) or tyrosine phosphatases (e.g., DUSP3) may yield protection from atherothrombotic complications. Other potential targets include molecules such as PDI, which function at the intersection of blood coagulation and platelet activation. Carefully performed clinical trials are then required to validate these targets in larger patient cohorts. Finally, the immune component in arterial thrombosis warrants further characterization. Activated monocytes and neutrophils not only participate in a mutual interplay with platelets, which potentially results in reciprocal activation, but also have direct impact on thrombus formation. These recent findings open up new therapeutic avenues in research and contribute to the development of safer strategies in arterial thrombosis.

Take Home Messages

- Platelets are key players in arterial thrombosis.
- Inhibition of platelet function reduces thrombus formation but is associated with an increased risk of bleeding.
- Antiplatelet strategies targeting thrombotic functions, but leaving hemostasis unaffected, are warranted.
- Novel platelet signaling pathways and surface receptors more specifically modulating thrombotic processes have been identified, which could lead to new therapies.
- Innate immune cells contribute to arterial thrombosis, opening up additional avenues of research.

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Platelets in Atherosclerosis

Madhumita Chatterjee and Meinrad Gawaz

Abstract

Platelets play a critical role in haemostasis and thrombosis. Recent discoveries provide evidence that platelets promote atherogenesis and augment vascular inflammation and remodelling of the arterial wall, resulting in formation of atherosclerotic plaques. Upon activation, platelets adhere to the endothelial monolayer and release a variety of inflammatory mediators. Platelet-derived inflammatory mediators promote activation of the endothelial monolayer and recruitment of circulating blood cells, including monocytes and endothelial progenitor cells (EPCs). Further, platelets stimulate differentiation of monocyte and endothelial progenitor cells into macrophages and foam cells. Thus, at the site of platelet accumulation on the arterial wall, platelets form an inflammatory 'hot spot' that constitutes an early trigger for atherosclerotic lesions. On the other hand, platelets also control healing of vascular lesions by recruitment and differentiation of circulating endothelial progenitor cells to promote repair of the endothelial monolayer. Thus, platelets ensure both repair of vascular lesions ('healing') and foster lesion formation and progression, depending on atherosclerotic cofactors such as oxidized LDL (oxLDL). The diverse mechanisms by which platelets accomplish a balance between vascular injury and repair encompass a variety of inflammatory mediators and receptors. Recent clinical studies provide evidence that an intensified and prolonged antiplatelet therapy improves clinical prognosis of patients with atherosclerotic disease. This chapter highlights the recent developments concerning platelets in the context of atherosclerosis and highlights the novel therapeutic strategies.

Introduction

The pathogenesis of atherosclerosis encompasses a strong inflammatory component that involves various cell types including platelets and inflammatory mediators (Lusis 2000; Libby et al. 2011). Not long ago the pathophysiological contribution of platelets to atherosclerosis was contemplated mainly in the context of the coagulation cascade, participating in the final step of atherosclerosis, and eventual thrombus build up following plaque rupture causing thrombotic narrowing or occlusion of a vessel. However, abundant experimental evidences have changed this point of view and established platelets as early responders seeding atheroprogression. Platelets are the prime responders to vascular injury and ensure haemostasis and vascular integrity to prevent extravasal blood loss (Ruggeri 2002, 2009). Platelets rapidly adhere to vascular lesions via adhesion receptors (e.g. GPIb, GPVI), aggregate via the fibrinogen receptors $\alpha_{IIb}\beta_3$ and become activated during the adhesion process. Usually, the intact endothelial monolayer prevents platelet attachment and accumulation at the vessel wall. However, inflamed endothelial cells (ECs) develop properties that render them adhesive for platelets. In accordance with the 'response-to-

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injury' hypothesis of atherogenesis, atherosclerotic lesions result in an orchestrated response to a localized injury to the vascular endothelium, which subsequently triggers platelet adhesion, aggregation and release of pro-inflammatory mediators like regulated upon activation normal T-cell expressed and secreted (RANTES), epithelial neutrophil-activating peptide (ENA-78), IL-1β, chemotactic (e.g. stromal-derived factor-1, SDF-1) and growth factors (e.g. platelet-derived growth factor, PDGF) from activated platelets. Upon activation platelets also release substantial amounts of alarmin or danger signals which function as inflammatory mediators and *damage-associated molecular pattern* proteins (DAMPs) (Gleissner et al. 2008; Gawaz et al. 2005; Karshovska et al. 2013; Gawaz and Vogel 2013). Activated platelets recruit circulating blood cells (e.g. monocytes and endothelial progenitor cells) at the site of vascular lesions and interact with resident vascular cells such as endothelial and smooth muscle cells (Langer and Gawaz 2008). Under physiological conditions all these mechanisms contribute to vascular repair and preservation of vessel integrity.

Platelets adhere under pathophysiological conditions to endothelial cells via multiple adhesion receptors including selectins, integrins and immunoglobulin-type receptors (Gawaz 2004). During the adhesion process, platelets become activated and release an arsenal of potent inflammatory and mitogenic substances into the local microenvironment, thereby alter the characteristics of the endothelial monolayer into a proatherogenic substrate (Gleissner et al. 2008; Karshovska et al. 2013). Platelet-derived chemokines and DAMPs attract circulating monocytes and endothelial progenitor cells towards the inflamed endothelium, which is a critical step in atherosclerotic lesion formation (Karshovska et al. 2013; von Hundelshausen and Schmitt 2014; Chatterjee and Gawaz 2013). Once circulating blood cells are recruited towards the atherogenic *hot spot*, platelets induce differentiation of recruited bone-marrow-derived cells into macrophages/foam cells (Massberg et al. 2006; Stellos and Gawaz 2007a, b; Langer et al. 2007; Stellos et al. 2008, 2010; Daub et al. 2006). A key mechanism in this differentiation process is the generation and uptake of oxidized low-density lipoprotein (oxLDL). OxLDL is bound to the surface and phagocytosed by platelets by means of scavenger receptors like CD36, LOX-1 and CXCL16/PS-OX, making platelets a major vehicle for oxLDL (Fig. 1)

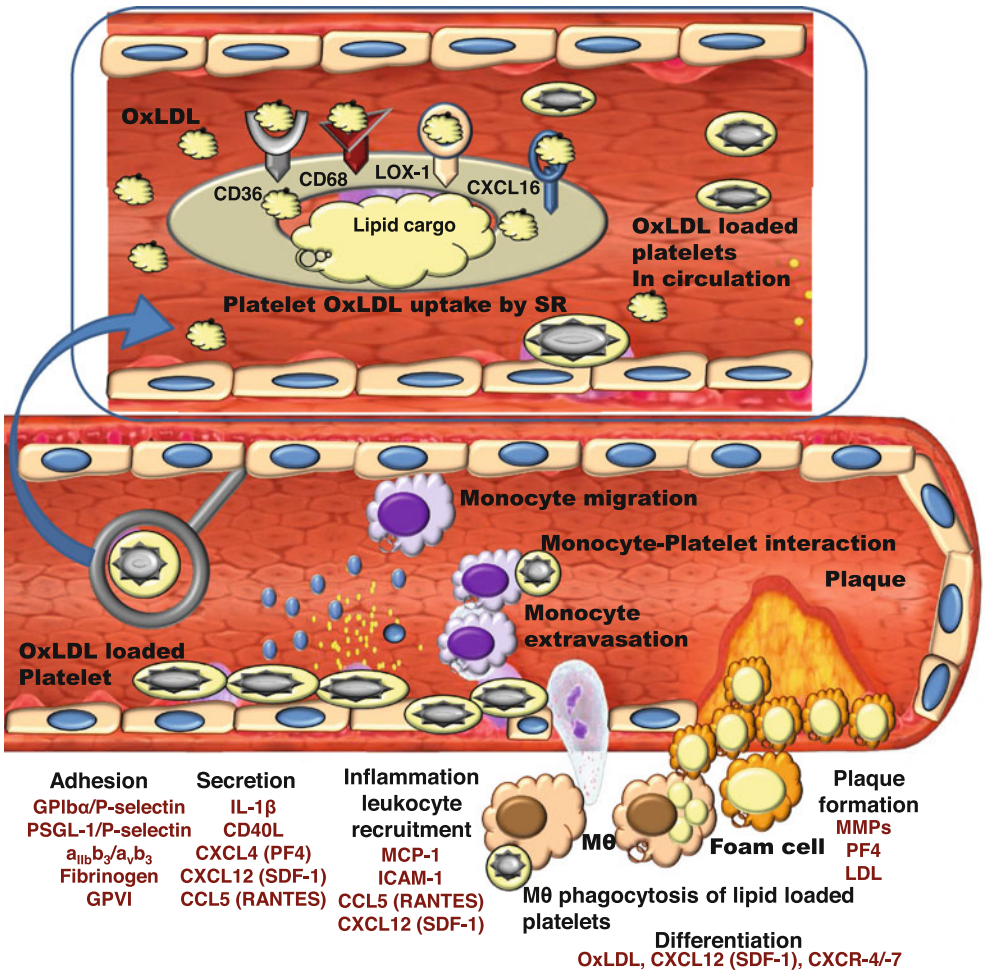

Fig. 1 Platelets orchestrate atherogenesis. Above: Platelets interact with circulatory lipoproteins. Platelets can take up oxLDL by scavenger receptors like CD68, CD36, LOX-1 and CXCL16/PS-OX. Therefore platelets serve as lipid vehicles in circulation. Below: Activated lipid-laden platelets in circulation interact with the vascular endothelium, release pro-inflammatory factors and also induce a proatherogenic phenotype of ECs. Subsequently, adherent platelets recruit circulating monocytes, bind them and inflame them by receptor interactions and paracrine pathways, thereby initiate monocyte transmigration, their phagoctytic uptake of lipid-loaded apoptotic platelets and differentiation into macrophages and foam cells. Thus, platelets provide the inflammatory basis for plaque formation before physically occluding the vessel by thrombosis upon plaque rupture

(Siegel-Axel et al. 2008; Magwenzi et al. 2015; Badrnya et al. 2014). OxLDL-rich platelets are rapidly taken up by monocytes and stimulate transformation into macrophages and foam cells (Stellos et al. 2010; Daub et al. 2006, 2007; Chatterjee et al. 2015a, b; Nurden 2011). Thus, platelet adhesion, release of inflammatory mediators, recruitment of monocytes, uptake of lipid-laden platelets by monocytes and their differentiation into foam cells represent a critical step in early lesion formation of atherosclerotic plaques (Fig. 1). On the other hand, platelet interaction and phagocytosis by endothelial progenitor cells can induce differentiation into an endothelial phenotype that favours vascular repair and regeneration and may limit atherosclerotic lesion progression (Massberg et al. 2006; Stellos and Gawaz 2007a, b; Langer et al. 2007; Stellos et al. 2008, 2010; Daub et al. 2006) (Fig. 2). The key mechanisms that flip the switch between regeneration versus disease progression remain unknown. Thus, understanding the mechanistic basis that modulates platelet-dependent vascular inflammation and formation of atherosclerotic lesions is an attractive strategy to prevent or to control the consequences of atherosclerosis such as myocardial infarction or stroke.

Platelet Adhesion to the Endothelium

Under pathophysiological circumstances platelets adhere even to the intact endothelial monolayer especially at the lesion-prone sites of vessel bifurcation governed by altered shear stress. Normal 'resting' endothelium represents a nonadhesive and non-thrombogenic surface that prevents extravasation of circulating blood cells. In contrast, activated endothelial cells are pro-adhesive and promote the adhesion of circulating blood platelets (May et al. 2008; Gawaz et al. 1991, 1996, 1998). Adhesion of platelets to the intact but activated endothelium in the absence of previous endothelial denudation involves a surface receptor-dependent process that allows *capturing* or tethering of circulating platelets towards the vessel wall even under high shear stress, followed by rolling and subsequent firm adhesion. Platelets are well equipped to adhere to endothelial cells in vitro and in vivo. Platelet interaction with intact endothelium is a well-orchestrated procedure (Fig. 3). These processes are dependent on receptor interactions via selectins, integrins and immunoglobulin-like receptors, which induce receptor-specific activation signals in both platelets and the interacting cell partner involved in adhesion, for instance, endothelial cells. Initially, platelets roll loosely on the endothelial layer. Rolling is often dependent on endothelial activation induced by inflammatory assaults inflicted by infection, mechanic erosion or ischaemia and reperfusion. High levels of C-reactive proteins are associated with high rate of vascular events and promote platelet adhesion to endothelial cells. Platelets get activated during rolling interaction with the endothelium and subsequently adhere more and more tightly. Endothelial cells in turn get activated, too, and both cells express or secrete chemokines to perpetuate vascular inflammation. Activated endothelial cells surface-express ICAM-1, vascular cell adhesion molecule-1, E-selectin and P-selectin and release the chemokines MCP-1, SDF-1 and interleukin-8. Both activated platelets and endothelial cells can actively release pro-inflammatory interleukin-1β and CD40L. Platelet-specific release is chiefly characterized as RANTES and ENA-78. This vast array of secretory products, besides mediating interaction with leukocytes and endothelium, acts as inflammatory cues for the recruitment of immune or inflammatory cells, e.g. monocytes, also prompt their differentiation into macrophages to seed the process of plaque formation and atherosclerosis. Rolling is followed by firm adhesion that is mediated by integrin binding. Firm platelet adhesion triggers maximal platelet activation, instigating shape change, cytoskeletal rearrangement and release of potent inflammatory and mitogenic mediators into the local microenvironment. GPIIb-IIIa ($\alpha_{IIb}\beta_3$) is the major integrin on platelets and plays a key role in platelet accumulation on the activated endothelium. Among the integrins expressed on the luminal side of endothelial cells, the vitronectin receptor ($\alpha_v\beta_3$) plays a crucial role in promoting platelet adhesion. Another adhesion molecule for the contact of platelets with the vascular wall is GPIb-IX. Taken together platelet–endothelial cell interactions involving selectins, integrins and immunoglobulin-like adhesion receptors perpetuate transcellular communications leading to a pro-inflammatory status of both endothelial cells and inflammatory cells recruited to the site of action, thus contribute to vascular inflammation and atherosclerosis.

The Role of Selectins The initial loose contact between circulating platelets and the vascular endothelium ('platelet rolling') is mediated by selectins, present on both endothelial cells and platelets (Frenette et al. 1995, 1998a, b; Massberg et al. 1998; Subramaniam et al. 1996). P-selectin (CD62P) is rapidly expressed on the endothelial surface in response to inflammatory stimuli. In addition, P-selectin is stored in platelet α-granules and can rapidly translocate to the platelet surface upon activation. Endothelial P-selectin has been demonstrated to mediate platelet rolling in both arterioles and venules in acute inflammatory processes, such as ischaemia/reperfusion. Further, P-selectin glycoprotein ligand 1 (PSGL-1) is expressed on platelets and can mediate platelet–endothelial interaction in vivo (Lam et al. 2011; Frenette et al. 2000). E-selectin, which is also expressed on inflamed endothelial cells, allows a loose contact between platelets and the endothelium in vivo (Frenette et al. 1998a, b). In line with the concept of endothelial inflammation as a trigger for platelet accumulation, the process of platelet rolling does not require previous platelet activation, since platelets from mice lacking P- and/or E-selectin roll as efficiently as wild-type platelets (Frenette et al. 1995).

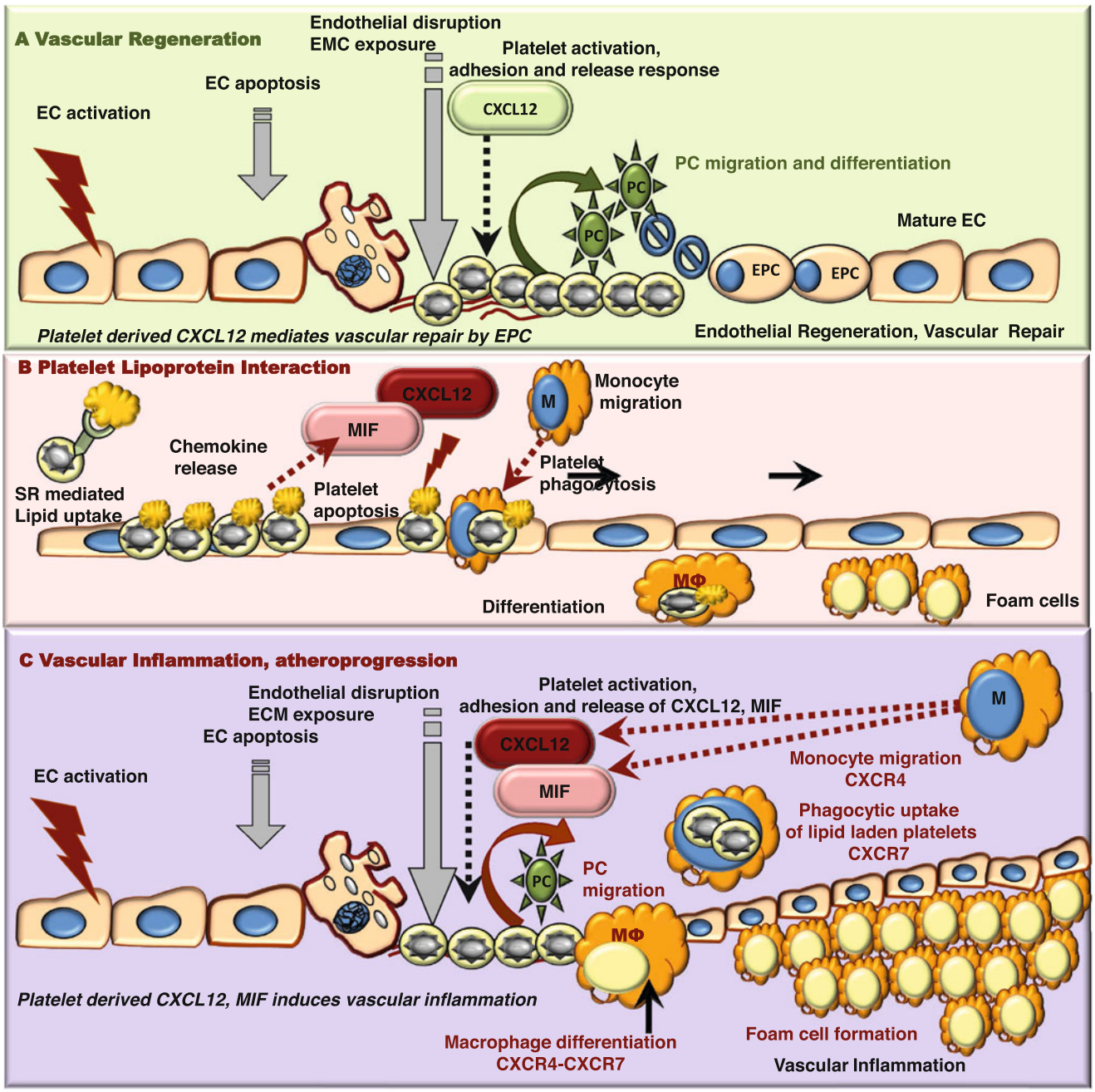

Fig. 2 Platelet chemokines in balancing atheroprogression and vascular regeneration. Platelets adhere to and interact with the injured or inflamed endothelium (EC, endothelial cell) or exposed sub-endothelial matrix components (EMC) and secrete CXCL12 and MIF upon activation. Platelet-derived CXCL12 mediates the migration and differentiation of progenitor cells (PC) into an endothelial phenotype (endothelial progenitor cell, EPC) to promote vascular repair or regeneration (**a**) and also supports the migration of inflammatory monocytic cells (M). Monocytes phagocytose activated platelets and CXCL12 present in the immediate microenvironment supports their differentiation into foam cells. This contributes to vascular inflammation and injury (**c**). Platelets bind lipids like LDL and OxLDL in circulation through scavenger receptors. Lipid-loaded activated platelets adhere to the endothelium and release chemotactic factors like CXCL12 and MIF. Monocytes migrate towards CXCL12 and MIF. These infiltrated monocytes phagocytose lipid-laden apoptotic platelets and subsequently migrate into the intima of the vessel wall where they differentiate into macrophages and foam cells (**b**). Thus platelet–lipoprotein interaction (**b**) and platelet-derived factors can decide the balance of vascular regeneration (**a**) versus vascular inflammation and atheroprogression (**b–c**)

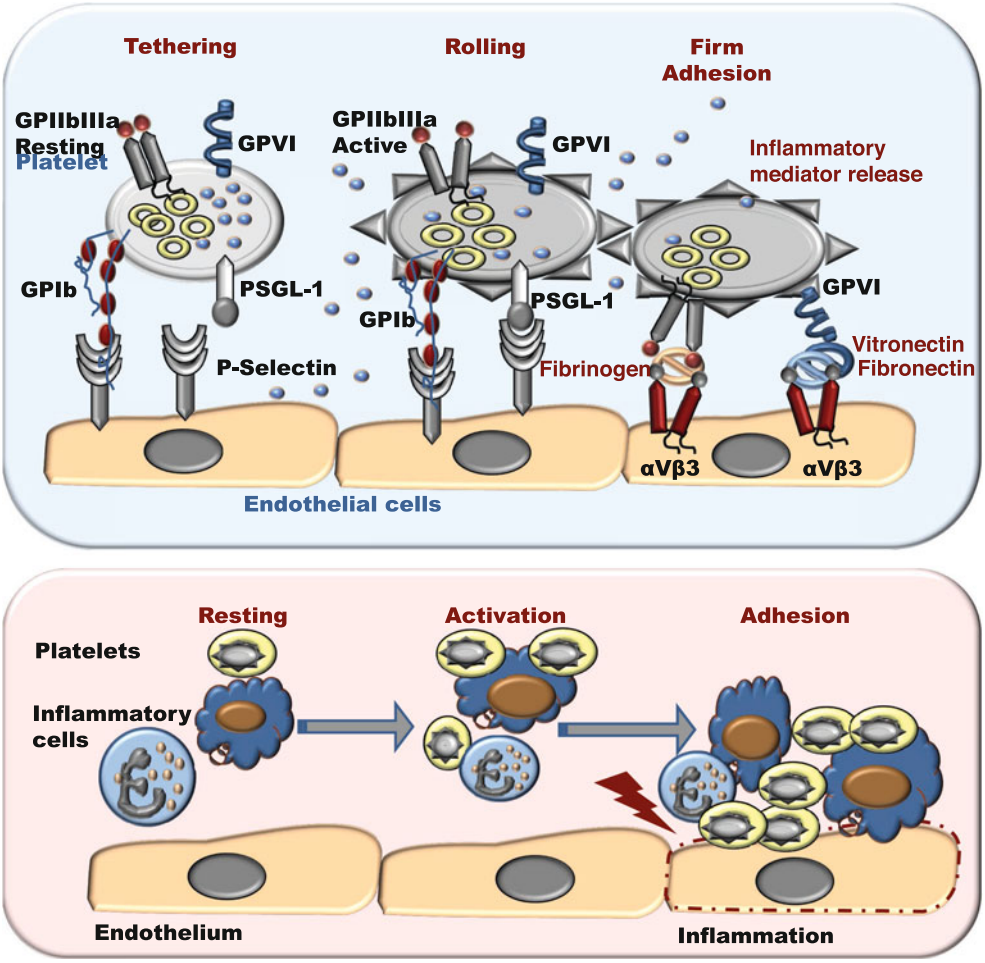

Fig. 3 Platelet interaction with the vascular endothelium. Above: Platelet–endothelium adhesion is a multistep process involving several adhesion receptors. Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIbα and PSGL-1 interact with endothelial P-selectin and mediate platelet rolling. Subsequent firm adhesion is mediated through β3 integrins. Below: Platelets recruit inflammatory cells like monocytes and neutrophils. Activated platelets in circulation form co-aggregates with inflammatory cells and foster their subsequent interaction with intact or inflamed endothelial cells and inflame monocytes. Thus, platelet–monocyte interaction provides an atherogenic milieu at the vascular wall that supports plaque formation

The Role of GPIb-IX-V Glycoprotein Ib (GPb) mediates platelet–endothelium adhesion (von Hundelshausen and Weber 2007). Glycoprotein Ib has been identified as counter-receptor for P-selectin (Etingin et al. 1993; Theilmeier et al. 2002). Platelet rolling on the activated endothelium can be inhibited by antibodies against both P-selectin and GPIbα (Frenette et al. 1995, 1998a, b; Massberg et al. 1998). Interactions of selectins with their counter-receptors are characterized by high on- and off-rates, enabling platelets to rapidly attach to the endothelial monolayer with high resistance to shear stress. However, due to their biophysical characteristics, selectin–ligand interactions are not sufficient to promote firm adhesion of platelets in the bloodstream. This implicates that these tighter interactions between platelets and the vascular wall involve the interplay of platelets and endothelial integrins as well as immunoglobulin-like adhesion molecules.

The Role of Integrins In the presence of soluble fibrinogen, $\alpha_{IIb}\beta_3$ mediates heterotypic cell adhesion to $\alpha_v\beta_3$-expressing cells including endothelial cells (Gawaz et al. 1991, 1996, 1997; Bombeli et al. 1998). Moreover, platelets firmly adhere to activated endothelial cells via $\alpha_{IIb}\beta_3$, a mechanism that can be blocked by antagonists of β3-integrins (Gawaz et al. 1997). In vivo, firm platelet adhesion to the endothelium can be inhibited by anti-$\alpha_{IIb}\beta_3$ mAb, and platelets defective in $\alpha_{IIb}\beta_3$ do not firmly adhere to activated endothelial cells (Gawaz et al. 1997).

The Role of Immunoglobulin-Type Receptors The intercellular adhesion molecule-1 (ICAM-1) is surface expressed on the inflamed endothelium and acts as an endothelial fibrinogen receptor, promoting fibrinogen deposition at the inflamed endothelium (Springer 1994). ICAM-1-fibrinogen interactions have been demonstrated to promote cell adhesion to activated endothelial cells (Bombeli et al. 1998). Another immunoglobulin-type platelet receptor GPVI mediates platelet adhesion to immobilized collagen, fibronectin and vitronectin, the matrix proteins which are exposed upon erosion of the inflamed endothelium (Bültmann et al. 2010; Schönberger et al. 2012).

The Role of Platelet Adhesion Receptors in Atheroprogression With the help of appropriate atherosclerotic animal models, it has become evident that platelets

adhere to the arterial wall early in the process of atherosclerosis before atherosclerotic plaques are morphologically detectable (Massberg et al. 2002, 2005; Huo et al. 2003). Bone marrow transplantation experiments demonstrated that mice receiving P-selectin-deficient platelets develop smaller lesions (Burger and Wagner 2003). Even more drastic findings were obtained in a model of wire-induced arterial injury in *P-selectin*$^{-/-}$*Apoe*$^{-/-}$ double knockout mice (Manka et al. 2004). The most striking effects in inhibiting atherosclerosis could be achieved with a combined deficiency of E-selectins and P-selectins, showing 80 % and 40 % protection in the early and advanced stages of the disease (Dong et al. 1998). Another critical adhesion molecule mediating platelet contact with the vascular wall is GPIb-IX, the significance of which is exemplified by the fact that prolonged antibody blockade of platelet GPIbα profoundly reduced leukocyte accumulation in the arterial intima and attenuated atherosclerotic lesion formation (Massberg et al. 2002). The contribution of platelet GPIIb (α_{IIb}) was also validated in atherosclerotic lesion formation in the carotid artery and aortic arch among *GPIIb*$^{+/+}$*Apoe*$^{-/-}$ and *GPIIb*$^{-/-}$*Apoe*$^{-/-}$ mice where the absence of GPIIb attenuated lesion formation at both vascular locations (Massberg et al. 2005). Further, administration of activated platelets and platelet–leukocyte/monocyte aggregates in *Apoe*$^{-/-}$ mice promote formation of atherosclerotic lesions (Huo et al. 2003). In *Apoe*$^{-/-}$ mice, platelet adhesion to atherosclerotic arteries was significantly inhibited by i.v. injection of soluble GPVI-Fc (Schulz et al. 2008; Schonberger et al. 2008; Ungerer et al. 2013). Long-term administration of an anti-GPVI antibody attenuates atherosclerosis in *Apoe*$^{-/-}$ mice (Schulz et al. 2008). Further, gene transfer of GPVI-Fc to the carotid vascular wall significantly attenuates atheroprogression and endothelial dysfunction in atherosclerotic rabbits in vivo (Bültmann et al. 2010). Inhibition of GPVI both via GPVI-Fc and anti-GPVI antibodies results in protection against atherosclerosis in both cholesterol-fed rabbits and *Apoe*$^{-/-}$ mice (Bültmann et al. 2010).

Platelet Interaction with Leukocytes

Activated platelets promote leukocyte arrest on the vascular endothelium, which is a key process of vascular inflammation during the progression of atherosclerosis (Lusis 2000; Libby et al. 2011). The interaction of platelets with leukocytes has been extensively described (Zarbock et al. 2007). Briefly, platelets physically interact with leukocytes (Rinder et al. 1991; Gawaz et al. 1994; Ott et al. 1996; May et al. 1997) and EPCs (Stellos et al. 2013). Platelet–leukocyte coaggregation can foster adhesion to the intact arterial wall. This interaction can occur in variable sequences: first, platelets can coaggregate with leukocytes in circulation and thereby support leukocyte recruitment to the endothelium by activating leukocyte adhesion receptors or by directly serving as bridging cells. For example, platelet–monocyte co-aggregates can attach to the vascular endothelium by both platelet and endothelium or by monocyte–endothelium contacts. Second, when adhered to the endothelium, platelets can chemoattract leukocytes and then provide a surface for their adhesion to the vascular wall. During these interactions involving platelets, leukocytes and the endothelium, all cell types involved become activated in a cascade-like manner (Fig. 3). Upon adhesion, platelets rapidly translocate P-selectin from α-granules to the plasma membrane. This allows leukocytes to tether to platelets via PSGL-1/P-selectin interaction. Subsequently, monocytes or polymorphonuclear cells firmly adhere to platelets in a Mac-1-dependent (CD11b/CD18, αMβ2) manner (Chavakis et al. 2003). On platelets, various counter-receptors of Mac-1 have been identified: GPIbα (Simon et al. 2000), junctional adhesion molecule-C (JAM-C, JAM-3) (Santoso et al. 2002), CD40L (Zirlik et al. 2007), ICAM-2 (Diacovo et al. 1994) as well as bridging proteins, such as fibrinogen (bound to $\alpha_{IIb}\beta_3$) (Altieri et al. 1988) or high-molecular-weight kininogen (Chavakis et al. 2003). However, the exact contribution of each receptor system awaits clarification. During this adhesive process, receptor engagement of PSGL-1 and Mac-1, together with platelet-derived inflammatory mediators, induces complex activation cascades in monocytes (Neumann et al. 1997; McEver and Cummings 1997; Weyrich et al. 1996) including NFkB activation and thereby promotes monocyte or neutrophil adhesion (by upregulation and activation of Mac-1 and VLA-4), thrombosis (mediated through monocyte secretion of tissue factor), monocytic chemokine and cytokine release (IL-1β, IL-8, MCP-1, TNF-α) (Neumann et al. 1997; McEver and Cummings 1997; Weyrich et al. 1996; Celi et al. 1994) or the oxidative burst of neutrophils (Zarbock et al. 2007). In addition, engagement of PSGL-1 by P-selectin also drives translationally regulated expression of proteins, such as the urokinase receptor (uPAR), a critical surface protease receptor and regulator of integrin-mediated leukocyte adhesion (May et al. 1998) in vivo. Additional adhesion receptor pairs also appear to be involved to induce vascular inflammation. For example, we have recently identified the extracellular matrix metalloproteinase (MMP) inducer (EMMPRIN, CD147) as a monocyte receptor that induces MMP-9 synthesis and secretion on cellular interactions (Schmidt et al. 2006; Lindemann et al. 2007). Thus multifaceted heterotypic platelet–endothelial cell, platelet inflammatory cell or progenitor cell interactions allow transcellular communication via soluble mediators inflicting pro-inflammatory damage to the vascular endothelium and subsequently recruit inflammatory cells to the site of atheroprogression (Fig. 4).

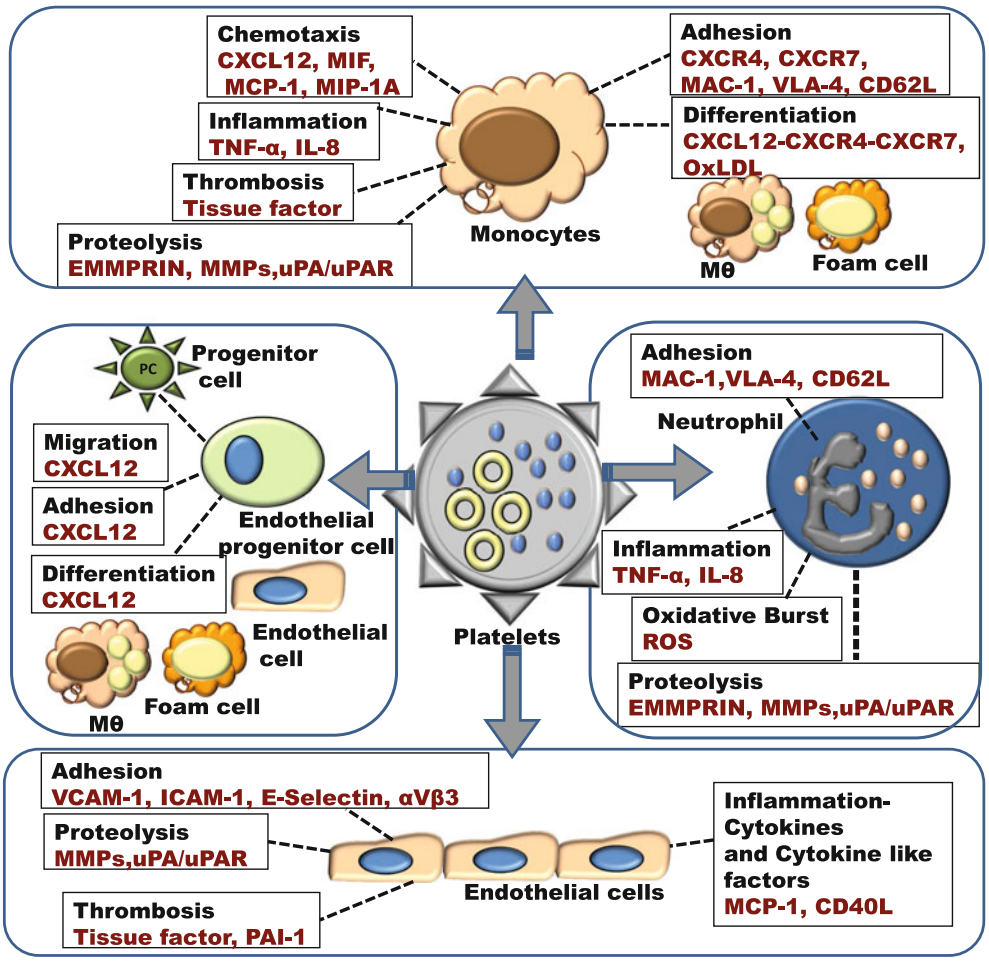

Fig. 4 Platelets have the ability to modulate atherothrombosis via interaction with other vascular cells. Adherent platelets inflame ECs. By adhesion to endothelial cells, platelets induce release of chemoattractants, upregulation of endothelial adhesion molecules and secretion of metalloproteinases. This is mediated by interference with the IκB and NF-κB pathway in ECs. Distinct receptor–ligand pairs mediate the interaction of platelets with endothelial cells involving $\alpha_{IIb}\beta_3$ induces platelet surface exposure of P-selectin (CD62P) and release of CD40L and IL-1β, which stimulate ECs to provide an inflammatory milieu that supports proatherogenic alterations of the endothelium. Platelet adhesion thereby contributes to atheroprogression, a process that involves complex and interacting steps, diverse cell types and mediators. Adherent platelets recruit and inflame monocytes. Adherent and/or activated platelets mainly interact with monocytic PSGL-1 via P-selectin and with monocytic Mac-1 (αMβ2) via $\alpha_{IIb}\beta_3$ (and fibrinogen bridging) or GPIbα. Thereby, platelets initiate monocyte secretion of chemokines, cytokines and procoagulatory tissue factor, upregulate and activate adhesion receptors and proteases and induce monocyte differentiation into macrophages. Thus, platelet–monocyte interaction provides an atherogenic milieu at the vascular wall that supports plaque formation. On the other hand, platelets serve as a bridging mechanism for circulating endothelial progenitor cells and can contribute to atheroprogression and vascular regeneration. Platelet derived inflammatory substances like CXCL12 along with potent adhesion molecules for circulating cells; platelets are capable of recruiting circulating endothelial progenitor cells (EPCs). Depending on the surrounding microenvironment, pro-atherogenic (for instance, the development of foam cells) or vascular reparatory mechanisms (differentiation of progenitor cells towards mature endothelial cells) can be promoted by interaction of platelets with progenitor cells

Platelet-Derived Inflammatory Mediators

Platelet–endothelium interaction occurs in the macro- and microcirculation of inflamed tissue and during reperfusion of ischaemic organs. Platelets adhere to the intact or inflamed endothelium or exposed sub-endothelial matrix components and release pro-inflammatory and mitogenic mediators including cytokines, chemokines and DAMPs that alter physiological characteristics of the vascular endothelium and either prompt tissue (Stellos and Gawaz 2007a, b) restoration or inflict vascular injury (Gawaz and Vogel 2013; Lindemann et al. 2007; Langer et al. 2007) (Fig. 2). Proteomic analysis of platelets has identified more than 300 inflammatory mediators including growth factors, chemokines, cytokines and angiogenic compounds that are released into the microenvironment of accumulating platelets (Maynard et al. 2007). Most of these inflammatory

mediators have been evaluated for paracrine cell function and are potent chemotactic compounds for monocytes. Some of the platelet-derived inflammatory mediators have been also shown to promote and modulate platelet function (*autocrine loop*) (Fig. 5). Atherosclerotic plaques are rich in chemokines including RANTES (Gear et al. 2001). Platelet-derived PF4 and RANTES immobilized on endothelial cells induce monocyte arrest on the activated microvascular or aortic endothelium (von Hundelshausen et al. 2005), promote survival of emigrated monocytes and their differentiation into macrophages (Scheuerer et al. 2000). Atherosclerotic plaque is enriched with CXCL12 deposited from adherent platelets, and a counter-regulation between CXCL12-driven domiciliation of endothelial progenitors (Massberg et al. 2006; Stellos et al. 2008) and PF4–RANTES-triggered recruitment of monocytes (Scheuerer et al. 2000) might influence the outcome of vascular regeneration as opposed to atheroprogression. Other CXC chemokines like fractalkine (Schäfer et al. 2004) and CXCL16 (Borst et al. 2012; Seizer et al. 2011) can heighten atherothrombosis by prompting platelet activation, degranulation and adhesion at sites of atherosclerotic developments. Evidently, fractalkine and CXCL12 are upregulated in neointimal SMCs, which become luminally exposed after arterial denudation (Scheuerer et al. 2000), whereby platelet activation under the pro-inflammatory influence of SMCs might inflict further vascular damage. This part of the chapter summarizes the celebrated platelet-derived accomplices in atheroprogression and particularly highlights the recently unravelled contribution of MIF, CXCL12, CXCL16, HMGB1 and CyPA which have emerged as new molecular targets.

Cytokines Platelet activation results in the release of interleukin-1 (IL-1β) and CD40 ligand (CD40L) (Gawaz et al. 2000; Henn et al. 1998). Activated platelets rapidly synthesize IL-1β via an extranuclear polysomal translation mechanism (Lindemann et al. 2001). Platelet–IL1β induces activation of human endothelial cells and smooth muscle cells and augments neutrophil adhesion (Gawaz et al. 2002; Massberg et al. 2003). CD40L, which belongs to the TNF family is cleaved and released from activated platelets (Henn et al. 1998). Platelet–CD40L induces synthesis and secretion of chemokines, adhesion molecules and metalloproteinases in endothelial cells and promotes neutrophil adhesion. Engagement of $\alpha_{IIb}\beta_3$ on platelets upregulates CD40L and triggers CD40L-dependent matrix degradation by endothelial cells (May et al. 2002). Administration of activated wild-type platelets but not CD40L-deficient platelets stimulates atherosclerotic lesion formation (Lievens et al. 2010).

Macrophage Migration Inhibitory Factor (MIF) MIF is an inflammatory cytokine with chemokine-like functions that plays a role in atherosclerosis (Morand et al. 2006; Bernhagen et al. 2007; Strüßmann et al. 2013). Expression of the pleiotropic inflammatory mediator is enhanced and intricately

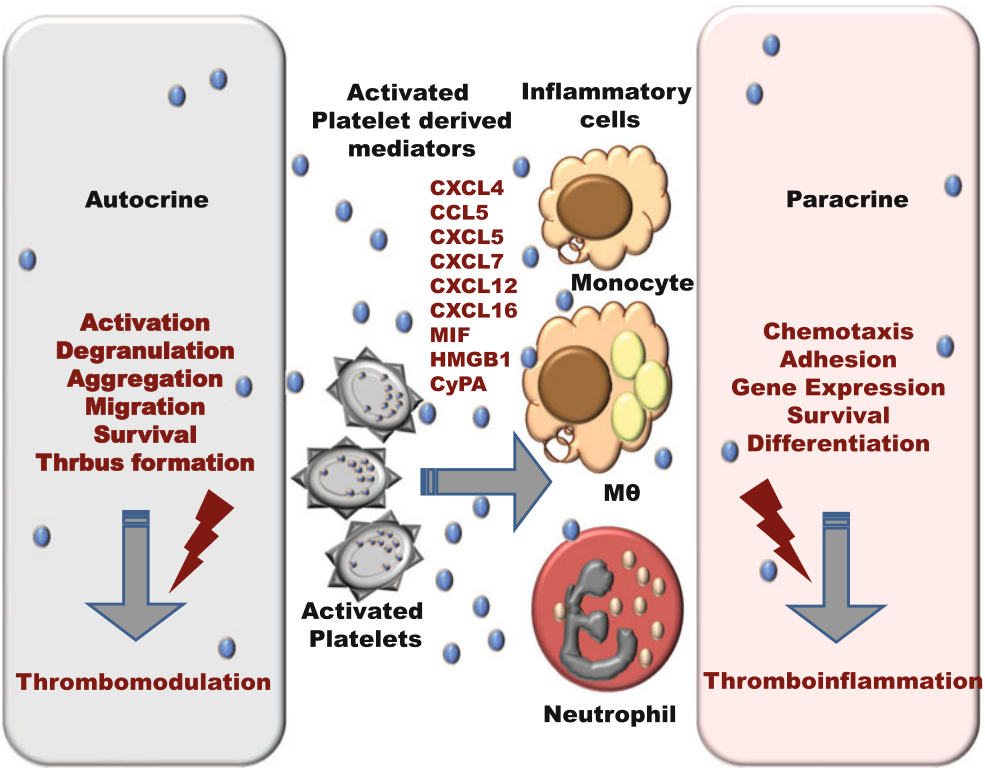

Fig. 5 Autocrine and paracrine effects of platelet-derived factors influence thrombomodulation and thromboinflammation. Platelet-derived inflammatory mediators play a critical role for thromboinflammation (paracrine) and thrombomodulation (autocrine), important mechanisms involved in vascular inflammation and atherosclerosis. Autocrine effects of platelet-derived CCL5, CXCL4, CXCL7, CXCL12, CXCL16, MIF, HMGB1 and extracellular CyPA modulate platelet activation, aggregation and thrombotic events, whereas in a paracrine mode of action, they influence chemotactic migration, adhesion, survival, differentiation and other pro-inflammatory attributes of inflammatory leukocytes by regulating expression of pro-inflammatory genes

associated with course of atherosclerotic progression. The therapeutic impact of MIF in atherosclerosis is exemplified by the fact that peripheral MIF depletion in *Apoe*$^{-/-}$ mice reduces atheroprogression (Bernhagen et al. 2007). MIF is a major platelet-derived chemotactic factor with a retarded release kinetics secreted by the nonclassical secretory pathway independent of ER–Golgi network, however results from degranulation of 60 % of total MIF reserve (Strüßmann et al. 2013; Wirtz et al. 2015) following thrombin and GPVI stimulation. MIF shows a diffused granular pattern of intracellular distribution but does not share co-localization with other α-granule constituents like PF4 and VEGF (Strüßmann et al. 2013). The chemotactic capacity of stimulated platelet supernatants is substantiated by MIF, suggesting a role for atherogenic cell recruitment (Strüßmann et al. 2013; Wirtz et al. 2015). The presence of neutralizing anti-MIF antibody significantly reduces the chemotactic potential of thrombin-activated platelet supernatant, whereas chemotactic potential of activated platelet supernatant derived from *mif*$^{-/-}$ mice is drastically reduced (Wirtz et al. 2015). Moreover, endothelial monolayers incubated with supernatants from MIF-enriched thrombin-stimulated platelets show significantly enhanced monocyte adhesion, supporting its potential as a platelet-derived pro-inflammatory mediator (Wirtz et al. 2015). MIF binds to several chemokine receptors including CXCR-2, CXCR-4 and CXCR-7 (Strüßmann et al. 2013; Wirtz et al. 2015; Chatterjee et al. 2014a, b; Alampour-Rajabi et al. 2015). Plasma levels of MIF are enhanced in acute coronary syndrome and associated with the inflammatory response (Müller et al. 2012, 2013, 2014). MIF binds to the DAN–protein gremlin-1, and a complex formation of MIF with gremlin-1 inhibits its chemotactic activity and reduces atheroprogression in *Apoe*$^{-/-}$ mice (Müller et al. 2013). Plasma levels of MIF and gremlin-1 are associated with acuity of coronary artery disease, and the MIF/gremlin-1 ratio might determine the grade of plaque stability in humans (Müller et al. 2014).

Chemokines The CC and CXC chemokines such as CCL5 (RANTES), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (β-TG), CXCL12 (SDF-1) and CXCL16 are the most intensively studied platelet-derived chemokines in the context of atherosclerosis. *CCL5 (RANTES)* is highly expressed and stored in platelets (Karshovska et al. 2013). Platelet–CCL5 is deposited on the endothelial monolayer and favours monocyte activation and adhesion (von Hundelshausen et al. 2001). CCL5 forms heteromers with *CXCL4 (PF4)*, and this complex synergistically supports monocyte/endothelium adhesion (Koenen et al. 2009). Administration of activated platelets in *Apoe*$^{-/-}$ mice enhances CCL5/CXCL4 deposition on the arterial wall and promotes atheroprogression via P-selectin (Huo et al. 2003). Plasma levels of CXCL5 and CXCL7 are increased in *Apoe*$^{-/-}$ mice (Rousselle et al. 2013). *CXCL5* enhances cholesterol efflux in macrophages and seems to be atheroprotective (Rousselle et al. 2013). *CXCL7* cleavage results in formation of platelet basic protein (PBP), beta-thromboglobulin (β-TG) and CTAPIII; all of these cleaved proteins are not chemotactic. After release those proteins are further activated by N-terminal shortening and promote migration of neutrophils and endothelial progenitor cells. *CXCL12 (SDF-1)* is stored in α-granules (Chatterjee et al. 2011) and gets surface expressed and released upon activation (Massberg et al. 2006; Stellos et al. 2008). Platelet–CXCL12 expression is increased in patients with myocardial infarction and correlates with circulating progenitor cells (Stellos et al. 2009). Platelet–CXCL12 and platelet surface expression of CXCR4–CXCR7 is associated with prognosis and recovery of myocardial function in patients with acute coronary syndromes (Geisler et al. 2012; Wurster et al. 2013; Rath et al. 2014, 2015). CXCL12 is a key mediator of regenerative mechanisms and regulates homing and trafficking of EPCs towards vascular and tissue lesions. Platelet–CXCL12 enhances neovascularization by mobilization of CXCR4^{+} cells in the mice hind limp ischaemia model (Jin et al. 2006) indicating recruitment of bone marrow-derived progenitors to support vascular repair. Platelet–CXCL12 interacts with CXCR4-positive EPCs and stimulates adhesion and endothelial differentiation. Systemic application of CXCL12 promotes mobilization of smooth muscle progenitor cells and accumulation in vascular lesions resulting in a stable plaque phenotype in *Apoe*$^{-/-}$ mice (Akhtar et al. 2013). Further, recombinant SDF1–GPVI triggers chemotaxis of CXCR4+ cells, preserves cell survival, enhances endothelial differentiation of BMCs in vitro and reveals proangiogenic effects. In a mouse model of myocardial infarction, administration of the bifunctional recombinant protein SDF1–GPVI leads to enhanced recruitment of BMCs, increases capillary density, reduces infarct size and preserves cardiac function (Ziegler et al. 2012). Thus, platelet–CXCL12 may be an atheroprotective chemokine which needs to be further explored. **CXCL16** is a multifaceted chemokine. Membrane-associated CXCL16/PS-OX is an OxLDL scavenger receptor which facilitates OxLDL binding to activated platelets, in its solubilized form exerts a stimulatory effect on their haemostatic and thrombotic attributes and also mediates inflammatory associations with the endothelium and eryptotic erythrocytes to corroborate atheroprogressive complications. Platelet–CXCL16 surface expression is further enhanced upon activation, and in ACS patients as compared to SAP, shows positive correlation with plasma C-reactive protein and markers of myocardial necrosis (Borst et al. 2012; Seizer et al. 2011). Recent results of a population-based HUNT2 cohort in Norway indicate that high levels of soluble CXCL16 is associated with risk of MI in healthy subjects (Laugsand et al. 2015). CXCL16 is detected in the human carotid atherosclerotic lesions from complex carotid endarterectomy specimens, sequesters circulating platelets through CXCR6 engagement and supports vWF-mediated platelet associations (Meyer Dos Santos et al. 2015). Moreover, high CXCL16 expression is

observed in the endothelium in close proximity to mural thrombus enriched in vWF and platelet GPIbα (Meyer Dos Santos et al. 2015). CXCL16 supplementation also supports platelet adhesion to the injured carotid artery in vivo (Borst et al. 2012). Platelets also release CXCL16 following PAR-1 activation by TRAP and therefore could contribute to CXCL16 plasma levels (Seizer et al. 2011). Moreover, since activated platelets release CXCL16 (Seizer et al. 2011), they might be an enriched source of circulatory CXCL16 levels among ACS patients, which serves as a peripheral biomarker and is associated with long-term motility. *CXCL16*$^{-/-}$ mice show decreased cholesterol efflux and attenuated atheroprogression. The role of platelet–CXCL16 however remains unclear and megakaryocyte–platelet-lineage-specific *CXCL16*$^{-/-}$ needs to be developed.

DAMPs Damage-associated molecular pattern molecules (DAMPs) are a heterogeneous group of nuclear or cytosolic host proteins that can initiate and perpetuate a noninfectious inflammatory response. DAMPs are released or exposed on the cell surface following tissue injury. Recently, two DAMPs have been recognized in platelets which contribute to regulation of thromboinflammatory events, namely, cyclophilin A (CypA) (Coppinger et al. 2004; Seizer et al. 2014, 2015; Elvers et al. 2012) and high-mobility group box 1 (HMGB1) (Rouhiainen et al. 2000; Ahrens et al. 2015; Vogel et al. 2014, 2015a, b). Platelet-bound CypA is enhanced in stable CAD patients, and its surface expression is associated with hypertension and hypercholesterolemia (Seizer et al. 2015). In patients with acute myocardial infarction (AMI), platelet-bound CyPA is significantly decreased (Seizer et al. 2015). CyPA stimulates vascular smooth muscle cell migration and proliferation, endothelial cell adhesion molecule expression and inflammatory cell chemotaxis (Seizer et al. 2015). *Apoe*$^{-/-}$ mice develop more severe atherosclerosis compared with *Apoe*$^{-/-}$/*CypA*$^{-/-}$ mice (Nigro et al. 2011). Platelets are a recently recognized source of high-mobility group box 1 (HMGB1) (Rouhiainen et al. 2000; Ahrens et al. 2015; Vogel et al. 2014, 2015a, b), the circulatory levels of which are elevated in multiple inflammatory diseases (de Souza et al. 2012). Platelets express HMGB1, which is translocated towards surface following activation and subsequently released (Rouhiainen et al. 2000; Ahrens et al. 2015; Vogel et al. 2014, 2015a, b). HMGB1 expression is increased in macrophages and SMCs at atherosclerotic lesions and is implicated in the progression of plaque (de Souza et al. 2012). The contribution of platelet-derived HMGB1 to influence infiltration of inflammatory cells during atheroprogression remains to be seen. Activated platelets interfere with recruitment of mesenchymal stem cells to apoptotic cardiac cells via HMGB1/TLR-4-mediated downregulation of hepatocyte growth factor receptor MET (Vogel et al. 2014). Recently, using a platelet-specific *Pf4-cre-HMGB1*$^{-/-}$ transgenic mice, we have identified platelet-derived HMGB1 as a critical mediator of thrombosis and inflammation in vivo (Vogel et al. 2015a, b). HMGB1 regulates microvascular endothelial inflammation (Fiuza et al. 2003) and leukocyte recruitment (Venereau et al. 2013); platelet-derived HMGB1 instigates neutrophil extracellular trap (NET) formation (Maugeri et al. 2014) and thrombosis (Vogel et al. 2015a, b) suggesting probable inflammatory potential of platelet-derived HMGB1 in linking atheroprogression and atherothrombosis. These evidences encourage further investigations to validate the potential of platelet-derived HMGB1 in influencing the cellular composition of atherosclerotic plaque and thereby its immunogenicity and vulnerability. HMGB1 is highly expressed in platelet-rich human coronary artery thrombi (Ahrens et al. 2015) pointing towards a central role for HMGB1 in atherothrombosis, also suggesting the therapeutic potential of platelet-targeted anti-inflammatory therapeutic strategies for CAD patients.

Platelet-derived HMGB1 might confer heterotypic plaque cellularity rendering them vulnerable, which combined with a pro-thrombotic disposition is an active accomplice in atherothrombosis and subsequent ischaemic events.

Regulation of Platelet Function and Thrombosis by Platelet-Derived Inflammatory Mediators

Upon release platelet-derived compounds can mediate both paracrine and autocrine effects (Fig. 5). Whereas the role of platelet-derived inflammatory mediators for paracrine actions and tissue inflammation has been extensively investigated, the autocrine effects of these molecules on platelet function are far less understood. Platelets express several chemokine receptors like CXCR-4, CXCR-6, CXCR-7 which render them susceptible to autocrine modulation imposed by factors such as CXCL12, CXCL16 and MIF (Chatterjee et al. 2015a, b). Further, platelets express receptors for CypA and HMGB1 (e.g. EMMPRIN, RAGE, TLR4) that significantly modulate platelet functions (Fig. 6). **CXCL12** substantiates platelet aggregation and thrombosis (Abi-Younes et al. 2000; Walsh et al. 2014; Kowalska et al. 2000; Gear et al. 2001; Shenkman et al. 2004). CXCL12 enhances platelet activation through Gαi-coupled CXCR4 (Abi-Younes et al. 2000; Walsh et al. 2014; Kowalska et al. 2000; Gear et al. 2001; Shenkman et al. 2004), antagonizes adenylate cyclase activity and counteracts PGI_2 analogue-induced cAMP levels (Walsh et al. 2014) and further substantiates granular release, PLC activation triggering aggregation. CXCL12–CXCR4-induced primary phase of aggregation occurs through PI3K, whereas the secondary wave involves engagement of downstream tyrosine kinases

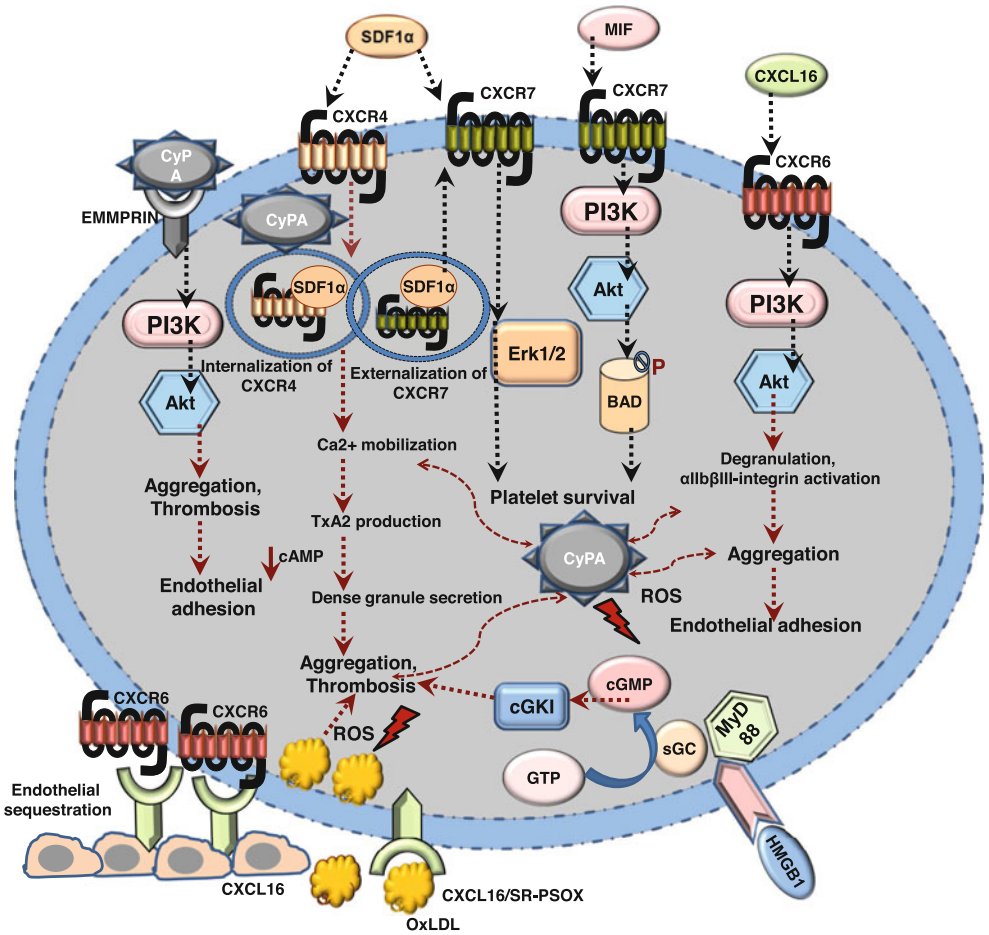

Fig. 6 New platelet-derived mediators in the context of atherothrombosis and atherosclerosis. CXCL12 through CXCR4 ligation substantiates platelet activation, aggregation and thrombotic potential. MIF exerts a prosurvival effect through CXCR7 and downstream activation of the PI3K–Akt pathway culminating in phosphorylation-mediated inactivation of pro-apoptotic protein BAD. The membrane-associated form of CXCL16/SR-PS-OX functions as a scavenger receptor facilitates OxLDL binding and sequesters CXCR6+ platelets from circulation to CXCL16-expressing endothelial cells at atherosclerotic sites. Soluble CXCL16 acts through CXCR6–PI3K–Akt pathway to promote degranulation, cytoskeletal reorganization and shape change, $\alpha_{IIb}\beta_{III}$-integrin activation and adhesion to endothelial layer. Intracellular cyclophilin A (CyPA) is involved in bidirectional trafficking of CXCR4–CXCR7 and extracellular CyPA acting through EMMPRIN engagement regulates aggregation, thrombotic potential of platelets. HMGB1 enhances platelet activation, dense and α-granule secretion, aggregatory response to GPVI stimulation and thrombus formation. HMGB1 exerts its effects through TLR4, myeloid differentiation factor 88-dependent (MyD88), recruitment of guanylyl cyclase (GC) towards platelet membrane and subsequent activation of cGMP-dependent protein kinase I (cGKI) in platelets

and prostanoids to achieve maximal irreversible aggregation and degranulation. CXCL12 co-stimulates aggregation induced by sub-threshold concentrations of agonists under arterial and lower shear conditions (Abi-Younes et al. 2000; Walsh et al. 2014; Kowalska et al. 2000; Gear et al. 2001; Shenkman et al. 2004) and exhibits selective synergistic aggregatory response with serotonin (5HT) but not epinephrine (Abi-Younes et al. 2000; Walsh et al. 2014; Kowalska et al. 2000; Gear et al. 2001; Shenkman et al. 2004). Chemokines are capable of heterophilic interactions exerting synergistic or antagonizing effects. RANTES noncompetitively reduces the stimulatory effect of CXCL12 on aggregation and adhesion to endothelial monolayers under venous flow conditions (Shenkman et al. 2004). Mature platelets also exhibit transmigration through endothelial layer towards CXCL12, mediated through CXCR4–Gαi–PI3K pathway which triggers platelet activation (Kraemer et al. 2010, 2011). Platelets preferentially accumulate at areas with high CXCL12 under flow conditions and exhibit flow-directed migration (Kraemer et al. 2010, 2011).

The receptors of CXCL12, CXCR4 and CXCR7 are constitutively expressed in platelets at transcript and protein levels. The relative surface expression of CXCR4 appears to be much higher than that of CXCR7 at resting state (Chatterjee et al. 2014b). Surface expression of CXCR4–CXCR7 exhibits a unique dynamism in the presence of their ligands, CXCL12 (Chatterjee et al. 2014b) and MIF (Chatterjee et al. 2014a) which influences their relative abundance on platelet surface,

thereby their frequency of participation in effector functions. CXCR4 is internalized by CXCL12 and MIF, while CXCR7 is preferentially externalized in response to CXCL12 but not MIF (Chatterjee et al. 2014b). CXCL12 induced bidirectional trafficking of CXCR4, and CXCR7 is a coupled process executed through the downstream signalling intermediates like Erk1/2 and the PPIase activity of intracellular molecular chaperone CyPA culminating in ubiquitination-driven externalization of CXCR7 (Chatterjee et al. 2014b). However, chemokine-induced receptor trafficking in platelets is a comparatively new idea and warrants further investigations in targeted pathophysiologies where platelet responsiveness and their inflammatory potential are altered. Moreover, CXCL12-executed effects on receptor trafficking can be influenced by the presence of other factors in the immediate microenvironment having differential binding affinities towards their cognate receptors (Chatterjee et al. 2014b). CXCL12 binds to CXCR4 on platelet surface with approximately 2000 sites/platelet and an affinity of 24 nmol/L but shares CXCR4 with MIF. Once released MIF can engage CXCR4 and CXCR7, while CXCR2 surface expression is relatively low and CD74 is absent on platelets. MIF ligates but does not influence CXCR7 availability on platelets. This discrepancy with CXCL12 is attributed to the absence of CD74 (which acts as a co-receptor for MIF–CXCR4 axis) and, therefore, lack of Erk1/2 activation downstream of CXCR4–MIF ligation, which is essential for CXCR7 externalization (Chatterjee et al. 2014a). At CXCL12/MIF-enriched atherosclerotic plaques, this dynamic receptor trafficking could influence relative CXCR4–CXCR7 availability with major functional implications. Both CXCL12 and MIF are ligands for the chemokine receptors CXCR4 and CXCR7 (Chatterjee et al. 2014a, b; Alampour-Rajabi et al. 2015). Unlike CXCL12, MIF does not seem to modulate platelet activation, degranulation or prompt release of chemokines (CXCL4, CXCL12) either alone or in combination with other agonists (Strüßmann et al. 2013; Wirtz et al. 2015). MIF does not amend aggregation by itself or that elicited by ADP and TxA2 analogue or influence integrin activation and spreading over fibrinogen (Strüßmann et al. 2013; Wirtz et al. 2015). MIF, unlike CXCL12, is unable to mobilize intracellular calcium pools in TxA2 receptor presensitized platelets; however, both CXCL12 and MIF significantly block/desensitize increases in calcium transient in response to ADP (Strüßmann et al. 2013; Wirtz et al. 2015). Thus, CXCL12 and MIF although sharing receptors show distinct effects on platelet functionality, possibly executed through distinct intracellular signalling cascades. However, MIF, like CXCL12, as a survival factor, rescues platelets from activation and BH3-mimetic induced apoptosis through CXCR7 engagement (Chatterjee et al. 2014a). The MIF–CXCR7-triggered anti-apoptotic effect is mediated through the PI3K–Akt pathway which culminates in phosphorylation-induced inactivation of pro-apoptotic effector BAD. CXCL12-induced surface availability of CXCR7 can mediate subsequent prosurvival benefits of both CXCR12 and MIF (Chatterjee et al. 2014a). Unlike CXCL12, as a prosurvival agent, MIF attenuates pro-thrombotic phosphatidylserine exposure on platelet surface, whereby MIF–CXCR7 also exerts an antithrombotic effect (Chatterjee et al. 2014a). Thus, current experimental evidence points towards a functional preference in executing haemostatic and thrombotic functions through CXCR4 (by CXCL12), while supports platelet survival through CXCR7 (by CXCL12 and MIF).

CXCL16/SR-PS-OX is a transmembrane chemokine which is cleaved and shedded from the plasma membrane via ADAM10 (Seizer et al. 2011). Soluble CXCL16 chiefly functions as a chemokine for inflammatory cells through CXCR6/BONZO (Borst et al. 2012). CXCL16 can induce platelet degranulation, $\alpha_{IIb}\beta_{III}$-integrin activation, cytoskeletal reorganization and shape change and adhesion to the endothelium under arterial flow conditions in vitro. CXCL16 enhances aggregatory response to sub-threshold ADP concentrations and fibrinogen (Borst et al. 2012). CXCL16 effects mediated through CXCR6 lead to downstream activation of PI3K–Akt pathway and are therefore significantly abrogated in $CXCR6^{-/-}$ and $Akt^{-/-}$ mice (Borst et al. 2012) and following pharmacological inhibition of the PI3K–Akt pathway. Moreover, CXCL16-triggered platelet activation is diminished in the presence of apyrase and purinergic P_2Y_1 and P_2Y_{12} receptor antagonists suggesting a feedback loop mediated through ADP (Borst et al. 2012). Immobilized or microsphere-bound CXCL16, resembling membrane-tethered version of the chemokine, enhances intracellular calcium mobilization and integrin activation, degranulation and aggregation responses to ADP (Borst et al. 2012; Meyer Dos Santos et al. 2015). Therefore, CXCL16 both as a soluble mediator and membrane-associated form can modulate activation and haemostatic functions of platelets.

The DAMP protein CyPA is an abundantly expressed intracellular protein which executes a variety of functions due to its PPIase activity. Intracellular CyPA participates in the bidirectional CXCR4–CXCR7 trafficking (Chatterjee et al. 2014b), whereas extracellular CyPA functions as a redox–stress-sensitive pro-inflammatory cytokine that contributes to atherosclerosis, cardiac hypertrophy myocardial infarction and myocarditis (Seizer et al. 2014). Intracellular CyPA also influences thrombotic and haemostatic functions (Elvers et al. 2012). $Cypa^{-/-}$ mice have prolonged bleeding time; exhibit impaired platelet degranulation, spreading, cytoskeleton reorganization, shape change and aggregation; and show attenuated thrombus formation despite comparable platelet counts and lack of severe haematologic abnormalities (Elvers et al. 2012). Therefore CyPA exerts dual effects in regulating platelet

function: it regulates Ca^{2+} signalling, and when released into the extracellular space, CyPA binds to its extracellular receptor CD147 (EMMPRIN) and thereby initiates a cascade of inflammatory processes. CyPA has been identified in the proteomic analysis of activated platelet releasate as one of >300 proteins, also detected at atherosclerotic plaques where platelets accumulate (Coppinger et al. 2004). Extracellular CyPA activates platelets via CD147/EMMPRIN-mediated PI3K–Akt, leading to enhanced platelet adhesion and thrombus formation in vitro even among *Cypa*$^{-/-}$ mice, independent of intracellular CyPA (Seizer et al. 2015). Thus platelet-derived CyPA is a potential therapeutic target in inflammatory cardiovascular ailments.

Another platelet–DAMP protein is HMGB1. Extracellular HMGB1 enhances platelet activation, dense and α-granule secretion, aggregatory response to GPVI stimulation, adhesion and spreading (Vogel et al. 2015a, b). Collectively, these functional modulations drive a pro-thrombotic disposition. HMGB1 exerts its effects through TLR4, myeloid differentiation factor 88-dependent (MyD88) recruitment of guanylyl cyclase (GC) towards platelet membrane and subsequent activation of cGMP-dependent protein kinase I (cGKI) in platelets. With platelets and pro-thrombotic aspects of HMGB1 come to light, the pathophysiological implication of platelet-derived HMGB1 needs to be evaluated in atherosclerosis (Vogel et al. 2015a, b).

In summary, platelet-derived inflammatory mediators play a critical role for thromboinflammation (paracrine) and thrombomodulation (autocrine), important mechanisms involved in vascular inflammation and atherosclerosis.

Effect of Low-Density Lipoproteins on Platelet Function

Lipoproteins are fundamental 'players' in atherogenesis since they change the properties of different cells involved in atherosclerosis and thrombosis. Low-density lipoproteins (LDL) and its oxidized form bind to the platelet surface via scavenger receptors SR-B, CD36, LOX-1 or CXCL16 (Siegel-Axel et al. 2008). Both oxLDL and LDL activate platelets and induce thrombus formation (Siegel-Axel et al. 2008). Platelets uptake and store both oxLDL and LDL in significant amounts in dense granules (Daub et al. 2007). Lipid-laden platelets are phagocytosed by macrophages (Daub et al. 2006) and induce foam cell development from monocytes and $CD34^+$ progenitor cells (Daub et al. 2006). The soluble scavenger receptor CD68 inhibits platelet-dependent foam cell generation in vitro (Daub et al. 2010) and atheroprogression in *Apoe*$^{-/-}$ mice (Zeibig et al. 2011). Further, oxLDL-laden platelets activate the endothelium, and the number of $CD34^+$ progenitor cells (colony-forming units), which would otherwise transform into endothelial cells, is significantly reduced in the presence of oxLDL loaded–platelets (Daub et al. 2007; Lindemann et al. 2007). Patients with ACS show significantly enhanced oxLDL binding on platelets as compared to patients with stable coronary CAD. Platelet-bound oxLDL positively correlates with the degree of platelet activation and plasma oxLDL levels. Preincubation of isolated platelets with OxLDL, but not with native LDL, results in enhanced platelet adhesion to collagen and activated endothelial cells under high shear stress in vitro, as well as after carotid ligation in *Apoe*$^{-/-}$ and wild-type mice (Stellos et al. 2012). Recently we found that oxLDL uptake by platelets induces platelet apoptosis, like other platelet agonists like thrombin and collagen-related peptide (CRP). CXCL12 facilitates phagocytosis of lipid-laden platelets by monocytes and M1–M2 macrophages and also promotes their differentiation into foam cells via CXCR4 and CXCR7 (Chatterjee et al. 2015a, b). Stimulation of platelets with oxLDL results in the formation of platelet–monocyte aggregates (PMA) and phagocytosis of platelets thereby increases oxLDL uptake by monocytes, dependent on platelet CD36 and CXCL4 release (Badrnya et al. 2014). Thus, platelets have the capacity to store and to transfer significant amounts of oxLDL to sites of atherosclerotic lesions as vehicles, which strengthens the significance of platelets in atherogenesis.

Platelet-Based Theranostics in Atherosclerosis

Apart from inflammatory mediators as highlighted in this chapter and summarized in Table 1, glycoprotein receptors like GPVI offer a platform to regulate platelet responsiveness, thrombotic propensity, inflammatory disposition and immune reactivity particularly in atheroprogression (Fig. 7). Platelet GPVI surface expression is enhanced following acute ischaemic events like myocardial infarction and cerebral stroke, thereby it serves as a biomarker and is associated with poor prognosis (Gawaz et al. 2014). Platelet adhesion to atherosclerotic lesions or ruptured plaques is primarily mediated through GPVI which favours atherothrombosis (Gawaz et al. 2014). Several diagnostic biomarkers have been developed in recent years for evaluation of thromboischaemic coronary and cerebrovascular diseases. These have significantly improved diagnosis and patient care and facilitated individualized risk assessment. A non-invasive platelet-based diagnostic and therapeutic strategy has emerged, which utilizes GPVI for lesion-directed antithrombotic therapy or to counteract atherosclerotic disposition. The objective of this approach is to ameliorate care of patients particularly in the context of cardiocerebrovascular medicine (Gawaz et al. 2014). Conventional imaging modalities to define atherosclerotic vessel disease and luminal stenosis have poor prognostic impact in

Table 1 Platelet-derived mediators contributing to atherosclerosis and atherothrombosis

Platelet-derived inflammatory mediator	Mediator	Receptor	Mechanisms effecting platelet biology	Refs.
Cytokines	Interleukin-1β	IL-1R	Induces activation of human endothelial cells and smooth muscle cells and augments neutrophil adhesion	Gawaz et al. (2000, 2002, 2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Lusis (2000), Magwenzi et al. (2015), Maugeri et al. (2014)
	CD40L[a]		Induces synthesis and secretion of chemokines, adhesion molecules and MMPs in endothelial cells and promotes neutrophil adhesion and matrix degradation by endothelial cells	Gawaz et al. (2005), Henn et al. (1998), Huo et al. (2003), Koenen et al. (2009), Lievens et al. (2010), Lindemann et al. (2007), Magwenzi et al. (2015), May et al. (1998)
	MIF[a]	CXCR-4 and CXCR-7	Anti-apoptotic, antithrombotic and atherosclerotic progression, plasma levels elevated in CAD and correlate with disease severity	Alampour-Rajabi et al. (2015), Chatterjee et al. (2014a), Müller et al. (2012, 2014), Naghavi et al. (2003), Theilmeier et al. (2002), Zarbock et al. (2007)
Chemokines				
CCL type	CCL-1 (I-309)	CCR8	Proatherogenic	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)
	CCL-2 (MCP-1)	CCR2	Proatherogenic, chemotactic. CCL2 presentation by platelets supports monocyte adhesion in vitro and neointima formation in vivo	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015), May et al. (1997)
	CCL-3 (MIP-1a)	CCR-1, CCR-2 and CCR-3	Proatherogenic, facilitates neointimal formation	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)
	CCL5 (RANTES)[a]	CCR-1, CCR-3 and CCR-5	Proatherogenic, facilitates neointimal formation, platelets deliver CCL5 (and CXCL4) to monocyte surface and the endothelium, resulting in increased leukocyte adhesion	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015), Massberg et al. (1998), Nurden (2011)
	CCL-7 (MCP-3)	CCR-1, CCR-2 and CCR-3	Proatherogenic	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)
	CCL-17 (TARC)[a]	CCR-4 and CCR-8	Synergistic platelet agonist, proatherogenic	Gawaz et al. (2005), Gear et al. (2001), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)
CXCL type	CXCL-1 (Gro-a)	CXCR-1 and CXCR-2	Support arrest of human monocytic cell lines and primary monocytes under flow conditions. Oxidative stress, eNOS downregulation in endothelial cells	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)
	CXCL-4 (PF4)	CXCR-3B	Synergistic platelet agonist, proatherogenic. EC activation (E-selectin expression). Induces monocyte activation (oxidative burst, induction of CCL3, CCL4 and CXCL8), macrophage differentiation, foam cell formation	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015), Scheuerer et al. (2000), von Hundelshausen and Schmitt (2014)
	CXCL-5 (ENA-78)	CXCR-2	Endothelial progenitor cell domiciliation, neutrophil chemoattractant	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015), Rousselle et al. (2013)
	CXCL-7 (β-TG, NAP-2)	CXCR-2	Endothelial progenitor cell domiciliation	Gawaz et al. (2005), Henn et al. (1998), Koenen et al. (2009), Lievens et al. (2010), Magwenzi et al. (2015)

(continued)

Table 1 (continued)

Platelet-derived inflammatory mediator	Mediator	Receptor	Mechanisms effecting platelet biology	Refs.
	CXCL-11 (ITAC)[a]	CXCR-7	CXCR7 internalization, survival	Chatterjee et al. (2014a)
	CXCL-12 (SDF-1)[a]	CXCR-4 and CXCR-7	Receptor internalization, activation, aggregation, pro-thrombotic, adhesion to immobilized collagen–fibrinogen, migration and survival. Atheroprogression, vascular regeneration and/or remodelling, PC mobilization, differentiation	Abi-Younes et al. (2000), Akhtar et al. (2013), Chatterjee and Gawaz (2013), Chatterjee et al. (2015b), Daub et al. (2006), Gear et al. (2001), Geisler et al. (2012), Jin et al. (2006), Langer et al. (2006), Massberg et al. (2006), Rath et al. (2014, 2015), Stellos et al. (2008, 2009, 2010, 2012), Stellos and Gawaz (2007a, b), Walsh et al. (2014), Wurster et al. (2013)
	CXCL-16[a]	CXCR-6	Platelet surface expression elevated in ACS, plasma levels enhanced, OxLDL binding, pro-thrombotic, expression increased in macrophages and SMCs at atherosclerotic lesions, contributes to progression of plaque	Borst et al. (2012), Laugsand et al. (2015), Meyer Dos Santos et al. (2015), Seizer et al. (2011)
DAMPs	HMGB1[a]	TLR-4 RAGE	Pro-inflammatory, pro-thrombotic, proatherogenic, present in intracoronary thrombi and atherosclerotic plaques. Anti-HMGB1 strategies prevent atheroprogression	Ahrens et al. (2015), Rouhiainen et al. (2000), Vogel et al. (2014, 2015b), von Hundelshausen et al. (2005)
	CypA[a]	EMMPRIN GPVI	Platelet activation, intracellular calcium mobilization, pro-thrombotic, CXCR4-CXCR7 trafficking, atherogenic	Elvers et al. (2012), Seizer et al. (2014, 2015, 2016)

[a]Autocrine effects on platelet functions are defined

predicting subsequent thromboischaemic complications since they are caused by rupture-prone vulnerable plaques (Naghavi et al. 2003) and luminal collagen exposure towards the direction of circulation that are hardly detectable by conventional imaging tools (Langer et al. 2008). The soluble dimeric form of GPVI (GPVI-Fc) exhibits high affinity to collagen (Massberg et al. 2004), competes with platelet GPVI for binding collagen and thereby prevents platelet adhesion onto collagen in vitro and in vivo (Massberg et al. 2004; Schulz et al. 2008; Schonberger et al. 2008). GPVI-Fc binds to collagenous components at the core of human atheromatous plaque (Gawaz et al. 2014; Naghavi et al. 2003; Langer et al. 2008; Massberg et al. 2004; Schulz et al. 2008; Schonberger et al. 2008) and to vascular lesions in mice. Therefore, thrombogenecity of atherosclerotic plaques may be detected by using labelled GPVI. Utilizing ^{124}I-GPVI-Fc and in vivo scintigraphy, a sensitive, non-invasive imaging method to detect thrombogenicity of vascular lesions has been developed (Schonberger et al. 2008; Bigalke et al. 2013). PET imaging reveals an increased uptake of ^{124}I-GPVI-Fc at atherosclerotic lesions in the aortic arch of high-fat diet-fed *Apoe*$^{-/-}$ mice (Bigalke et al. 2013). To allow for clinical translation, ^{64}Cu may be preferred to ^{124}I due to its improved spatial resolution and adequate half-life time for delayed PET studies (Gawaz et al. 2014). Similarly, after systemic administration of GPVI-Fc-FITC, increased signals were recovered from the sites of the injured carotid artery in *Apoe*$^{-/-}$ mice (Wadas et al. 2010; Bigalke et al. 2011). Targeted contrast-enhanced ultrasound (CEU) using target molecules that are conjugated to the surface of microbubble (MB) agents has evolved as a non-invasive imaging technique to evaluate atheroprogression, a method which provides high prospective for early risk stratification. Recently an ultrasound-guided molecular imaging with GPVI-targeted MB (MB_{GPVI}) has been utilized to detect atherosclerotic lesions in mice at the aortic arch and truncus brachiocephalicus (Metzger et al. 2015) following systemic administration of MB_{GPVI}.

GPVI critically influences atherothrombosis. It ensues thrombus growth and stability in a coordinated action with thrombin. Targeting the GPVI axis does not compromise physiological haemostasis, which is mainly ensured by the GPIb–vWF axis (Gawaz et al. 2014). GPVI-Fc owing to its preferential deposition at sites of injured vessels therapeutically blocks GPVI-binding sites on exposed collagen (Fig. 7) at atherosclerotic lesions (Gawaz et al. 2014; 143–145, 149)

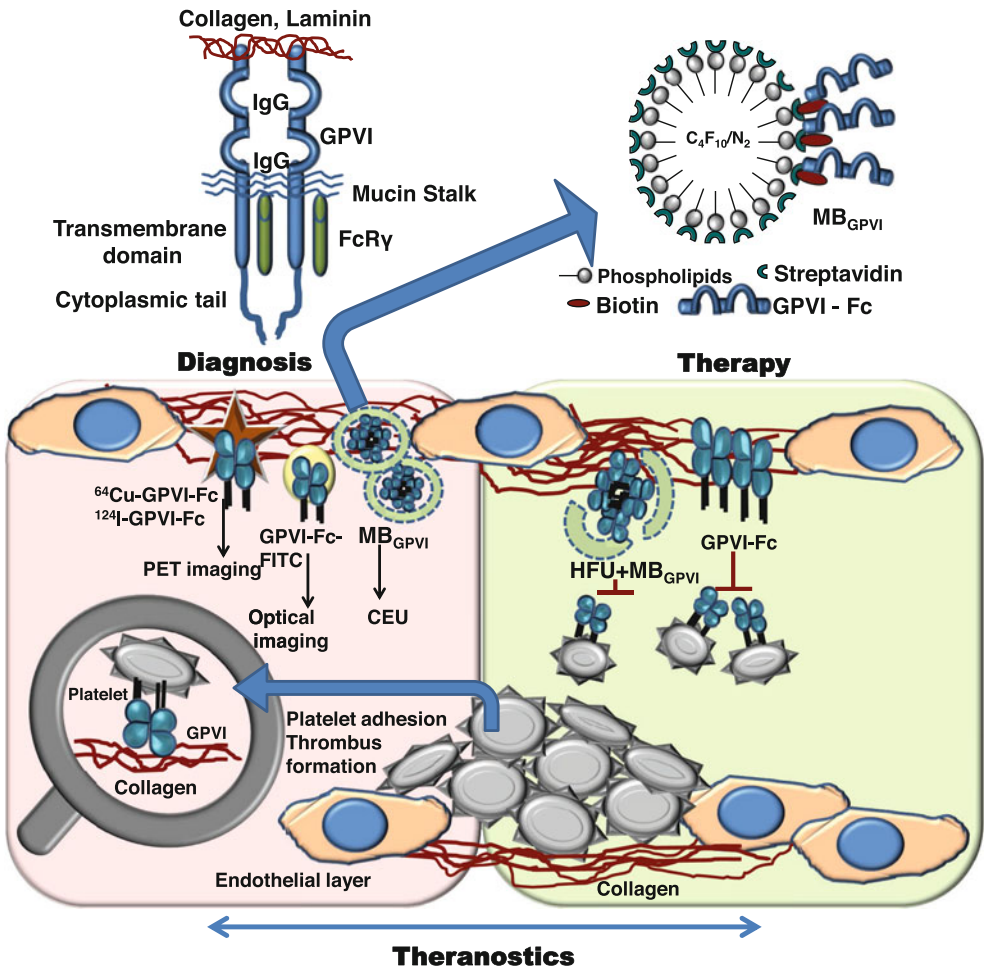

Fig. 7 Platelet theranostics to diagnose and combat atherosclerosis. GPVI is an ~62 kDa protein belonging to the immunoglobulin superfamily. The diagnostic and therapeutic potential of GPVI-based molecules combines to generate GPVI theranostics. Soluble dimeric GPVI-Fc can bind to extracellular matrix collagen in atheromatous plaque. Thrombogenecity of atherosclerotic plaques or non-invasive detection of vascular lesions at risk might be executed by using variously labelled GPVI derivatives like ^{124}I-GPVI-Fc, ^{64}Cu-GPVI-Fc, GPVI-Fc-FITC, GPVI-targeted microbubble agent (MB_{GPVI}) and their subsequent monitoring by PET imaging, optical imaging and contrast-enhanced ultrasound (CEU). Schematic representation of MB_{GPVI} is elaborated in the figure. MB_{GPVI} competes with platelet surface GPVI for binding collagen and attenuates platelet adhesion to immobilized collagen. High-frequency ultrasound (HFU)-guided disruption of MB_{GPVI} enables localized drug delivery at target site, significantly checks atherosclerotic development

and thereby counteracts stable arrest and aggregation of platelets without affecting bleeding times. Further, prolonged administration of GPVI-Fc attenuates atheroprogression (Gawaz et al. 2014) and arterial remodelling after mechanical injury in *Apoe*$^{-/-}$ mice. High-frequency ultrasound (HFU)-guided disruption of MB_{GPVI} injected in *Apoe*$^{-/-}$ mice by rapid ultrasonic 'burst' enhances GPVI accumulation at atherosclerotic lesions, covers the collagen-enriched surface therein, interferes with accumulation of GPIbα-positive platelets, checks lipid-rich plaque formation and exhibits noticeable reduction in plaque area limiting atherosclerotic development (Metzger et al. 2015). Therefore, GPVI-Fc, which ensures targeted delivery appears to be a promising diagnostic and therapeutic agent to combat atherothrombotic and inflammation involving platelet vascular interactions mediated through collagen and GPVI.

Does Uncontrolled Platelet Activation Promote Atherosclerosis in Humans?

There is convincing experimental evidence that platelets are a major driver of atherosclerosis. There are several supportive arguments that platelets initiate atherosclerotic lesions, foster atheroprogression and are critical in development of atherothrombosis leading to acute coronary or cerebrovascular events. Much clinical data has been published that link increased systemic platelet activation and hyperreactivity with poor clinical prognosis and clinical progression of coronary artery disease. Platelet reactivity is influenced by various clinical risk factors including diabetes mellitus, increased body mass index, left ventricular ejection fraction, renal failure, acute coronary syndrome, advanced age and

congestive heart failure (Geisler et al. 2008). Previously, a simple clinical risk score, *R*esidual *P*latelet Aggr*e*gation after *D*eployment of *I*ntra*c*oronary Sten*t* (PREDICT), was developed to identify patients with coronary artery disease at risk for increased platelet reactivity (Geisler et al. 2008; Droppa et al. 2015). The score encompasses different variables including acute coronary syndrome, older age, diabetes mellitus and renal and left ventricular function impairment. After weighing these variables according to their effects size in multivariate analysis, the score ranged from 0 to 9 with higher score levels being significantly associated with both platelet reactivity and cardiovascular outcome. Thus, comorbidity has a major impact on individual responsiveness to antiplatelet drugs. Clinical studies have shown that an increase in activation of circulating platelets is associated with the severity of coronary artery disease and progression of atherosclerosis (Gawaz et al. 2005). Platelet reactivity correlates with coronary plaque burden and calcification as assessed by cardiac computed tomography (Burgstahler et al. 2009). Further, systemic platelet activation is associated with progression of carotid artery disease in patients with diabetes (Fateh-Moghadam et al. 2005) and cardiac transplant vasculopathy (Fateh-Moghadam et al. 2000) within 1 year. Recently, the ADAPT-DES study showed that platelet reactivity is associated with atherosclerotic plaque burden and unstable plaque morphology in patients with coronary artery disease (Wang et al. 2016). In a clinical study, Trip and coworkers showed that spontaneous platelet aggregation in vitro is a useful biologic marker for the prediction of coronary events and mortality in survivors of a myocardial infarction (Trip et al. 1990). A recent systematic review and meta-analysis of individual patient data on major adverse cardiac events (MACE) outcomes (ACS, ischaemic strokes and vascular deaths) in relation to platelet reactivity and its interaction with cardiovascular risk levels were reported (Reny et al. 2015). 6478 patients, out of which 421 who experienced MACE (6.5 %) during a median follow-up of 12 months, were studied. The strength of the association between the risk of MACE and platelet reactivity increased significantly with the number of risk factors present (age $\geq$ 75 years, ACS at inclusion, diabetes and hypertension). Thus, it seems that the level of cardiovascular risk factors determines platelet reactivity and occurrence of MACE.

Currently, targeting cholesterol plasma levels in cardiovascular patients is the cornerstone in secondary prevention of atherosclerosis. To date statins and newly introduced PCSK9 inhibitors are established therapies for clinical progression of atherosclerosis. Besides lipid-lowering strategies, an intensified antiplatelet therapy is critical in treatment of patients with advanced atherosclerosis. Several oral antiplatelet drugs such as aspirin, $P2Y_{12}$ inhibitors (clopidogrel, prasugrel, ticagrelor) or PAR-1 antagonists (vorapaxar) as mono- or combination therapy are established treatment options for secondary prevention in patients with CAD. Since antiplatelet therapy not only reduces the thromboischaemic risk but also decreases ongoing platelet-driven systemic inflammation, possibly it also influences atheroprogression and occurrence of vulnerable plaques. Recent large clinical studies (PEGASUS, TRA2P, DAPT, OPTIDUAL) suggest that a prolonged and intensified antiplatelet therapy reduces progression of CAD as evidenced by reduction of ischaemic events, however, at the cost of higher bleeding event rates. Thus, targeting molecular mechanisms of thromboinflammation may turn out as innovative strategy for disease control among cardiovascular patients in the future.

Take-Home Message

Platelets accumulated at the site of vascular lesion or lesion-prone areas form a regulatory 'hot spot' that determines trigger for atherosclerotic lesions, plaque instability and subsequent atherothrombotic vessel occlusion.

Platelet–endothelial and inflammatory cell interactions mediated through ever-expanding array of adhesion molecules, pro-inflammatory, mitogenic factors and DAMPs promote atherogenesis and vascular remodelling.

Platelets recruit progenitor cells to the injured endothelium and platelet-derived mediators determine the balance between vascular inflammation and regeneration.

Intensified and prolonged antiplatelet therapy improves clinical prognosis of patients with atherosclerotic disease.

Advanced diagnostic and therapeutic strategies target platelet inflammatory biomarkers and mediators to diagnose atherosclerotic predisposition and prevent subsequent thromboischaemic complications.

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Platelets in Acute Coronary Syndromes

Mark R. Thomas and Robert F. Storey

Abstract

It has long been recognised that platelets play a key role in the formation of a thrombus after atherosclerotic plaque rupture. If the thrombus becomes occlusive, this may lead to impairment of the blood supply to the heart and an acute coronary syndrome.

Exposure of platelets to subendothelial components initiates platelet adhesion, mediated by the interaction of VWF with platelet GPIb-V-IX. The collagen receptors $\alpha_2\beta_1$ and GPVI then facilitate adhesion and mediate firm adhesion to collagen, respectively. Soluble agonists that are released also activate platelets, triggering specific intracellular signalling pathways and resulting in calcium mobilisation, shape change and increased expression and activation of the integrin $\alpha_{IIb}\beta_3$. This results in fibrinogen cross-linking activated $\alpha_{IIb}\beta_3$ on adjacent platelets, which mediates platelet aggregation.

Inhibition of specific platelet receptors has proved to be an exceptionally successful approach in the treatment of acute coronary syndromes. Although a number of receptor antagonists have been investigated, many receptors have still not been targeted in patients. This book chapter discusses the role of platelets in acute coronary syndromes, but in particular focuses on these potentially exciting, but as yet unexploited mechanisms.

Introduction

Acute coronary syndrome (ACS) refers to a group of conditions that arise when there is acute obstruction to blood flow in the coronary arteries, leading to an impairment of the blood supply to the myocardium of the heart. ACS is subdivided on the basis of electrical abnormalities detected on the electrocardiogram and detection of myocardial proteins that are indicative of myocardial infarction (Roffi et al. 2016). Partial obstruction often leads to unstable angina or non-ST-elevation MI (NSTEMI), whereas total obstruction of the larger coronary arteries normally leads to ST-elevation myocardial infarction (STEMI).

The recognition of the importance of platelets in ACS has led to some of the most successful pharmacological therapies in cardiovascular medicine. Coronary artery disease may not cause symptoms for decades, and the formation of an intracoronary thrombus by platelets is the pivotal moment that initiates ACS, which can be fatal (Falk et al. 2013). Platelets are involved in advanced atherosclerosis and their role intensifies following atherosclerotic plaque rupture or erosion. This exposes platelets to multiple thrombogenic substrates, such as collagen, von Willebrand factor (VWF) and fibronectin, to which platelets adhere, mediated by glycoprotein (GP) Ib-V-X, $\alpha_2\beta_1$ and GPVI. This initiates platelet activation and cross-linking of fibrinogen between platelet $\alpha_{IIb}\beta_3$ receptors, which causes platelet aggregation. Platelet activation is amplified by platelet release of dense granules and interactions with the coagulation cascade and leukocytes. Of these responses, platelet release of adenosine diphosphate (ADP) from dense granules and subsequent activation of platelet $P2Y_{12}$ receptors has a particularly

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important role in amplifying the response of platelets to other agonists.

Aspirin was the first medication to demonstrate the dramatic benefits of antiplatelet therapy in patients with ACS, which has led to the now routine strategy of dual antiplatelet therapy (Grove et al. 2015). However, patients with ACS continue to have increased risk of mortality, highlighting the need for further refinement of treatment strategies (Thomas and Storey 2015a). This chapter therefore discusses the major roles of platelets in acute coronary syndromes, with a particular focus on mechanisms that are potentially appealing novel pharmacological targets.

Platelets and the Pathogenesis of Acute Coronary Syndromes

Platelets appear to have a limited role in early atherogenesis (West et al. 2014), but play an increasingly important role during the lead up to atherosclerotic plaque rupture (Li et al. 2012) (Fig. 1). The development of an advanced atherosclerotic plaque is characterised by chronic inflammation, driven primarily by macrophages and T cells (Falk et al. 2013). There is increasing evidence that rupture and erosion of advanced plaques are relatively frequent and do not result in ACS unless other thrombogenic factors are present (Arbab-Zadeh et al. 2012). Multiple asymptomatic plaque ruptures and erosions appear to lead to an increase in plaque volume and the development of high-grade coronary arterial stenosis, however (Arbab-Zadeh et al. 2012). Throughout these processes, platelets promote the underlying inflammatory processes by direct interactions with the cells involved or by releasing pro-inflammatory cytokines (Thomas and Storey 2015b).

Platelets Interact with Activated Endothelium, Promoting Monocyte Recruitment

Although platelets do not normally adhere to endothelium that is intact, they are capable of adhering to activated endothelium (Massberg et al. 2002). Many conditions related to atherosclerosis, such as hypercholesterolaemia, smoking and oxidative stress, induce endothelial dysfunction and activation, resulting in expression of adhesion molecules, such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and E-selectin (Liao 2013). Additionally, atherosclerosis increases endothelial VWF, further promoting platelet adhesion and activation (De Meyer et al. 1999). Adhesion of platelets to these adhesion molecules and the endothelium is mediated by a variety of corresponding platelet adhesion molecules and receptors, including P-selectin, GPIbα, GPVI and $\alpha_{IIb}\beta_3$ (Massberg et al. 2002; Huo et al. 2003; Bültmann

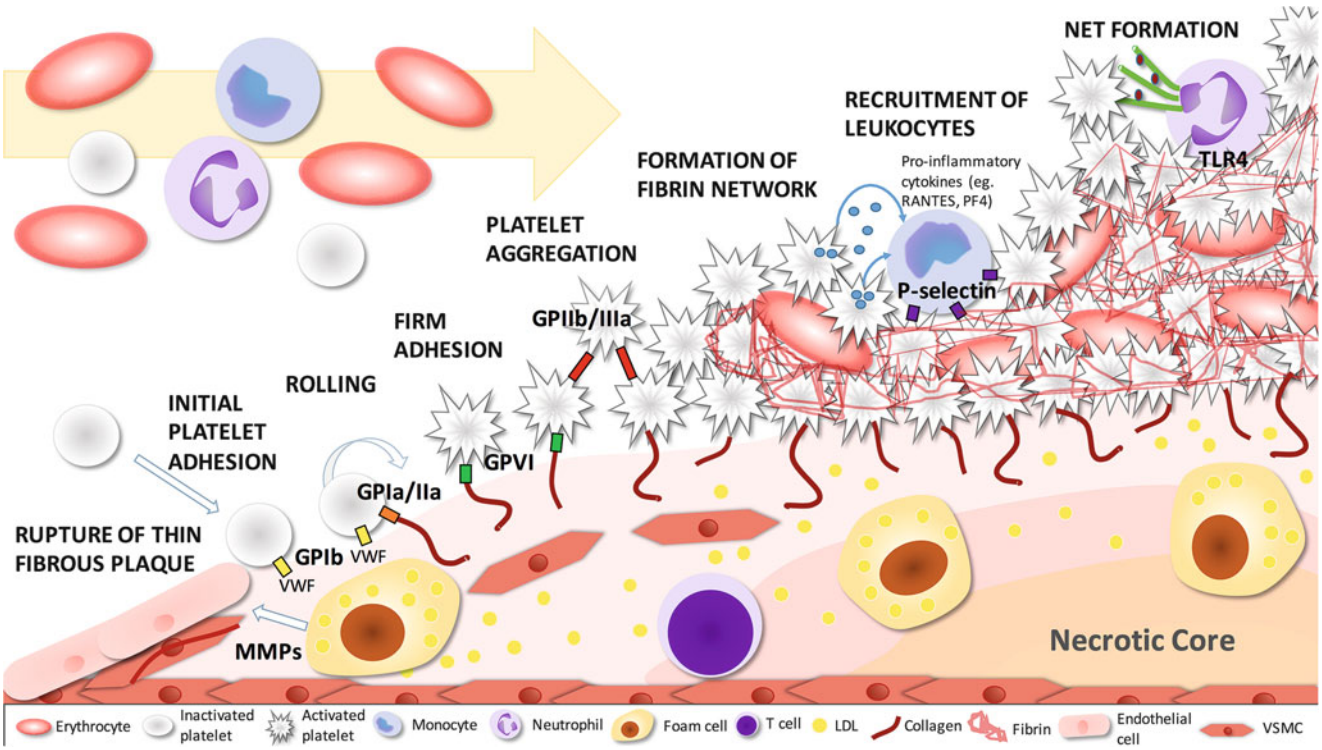

Fig. 1 The role of platelets in forming a thrombus following atherosclerotic plaque rupture

et al. 2010). Depletion of these adhesion receptors reduces leukocyte accumulation within the arterial vessel wall and reduces plaque formation in animal models (Massberg et al. 2002; Huo et al. 2003; Bültmann et al. 2010), demonstrating an important role of platelet adhesion in the pathophysiology of advanced atherosclerotic plaques.

Adherent platelets form bridges between the endothelium and leukocytes (Thomas and Storey 2015b), which facilitates the recruitment of monocytes to the atherosclerotic plaque where they take up cholesterol and become a type of macrophage known as a foam cell (Randolph 2014). Activated platelets release cytokines (Table 1), including platelet factor (PF) 4 and regulated on activation, normal T cell expressed and secreted (RANTES), which are released from α-granules and also act synergistically to promote recruitment of monocytes to the endothelium (Hundelshausen et al. 2005). Platelets are a major source of both PF4 and RANTES, and mice that are deficient in either PF4 or RANTES demonstrate attenuated development of atherosclerosis and reduced macrophage infiltration into atherosclerotic plaques (Koenen et al. 2009). Activated platelets also promote leukocyte recruitment by upregulating endothelial expression of ICAM-1 and release of monocyte chemotactic protein (MCP)-1 (Thomas and Storey 2015b). In addition, platelets facilitate T cell adhesion to the endothelium, and $CD4^+$ T cells actually appear to be incapable of sustaining adhesion with the endothelium under arterial flow conditions in the absence of platelets (Solpov et al. 2006). This enhancement of lymphocyte adhesion by platelets is mediated by P-selectin, $\alpha_{IIb}\beta_3$ and CD40L (Spectre et al. 2012). Platelets interact directly with monocytes and macrophages that are present in the atherosclerotic plaque to alter their phenotype and function. Platelet-derived PF4 prevents monocyte apoptosis and induces a phenotype in macrophages that is functionally distinct to both M1 and M2 macrophages and displays altered matrix metalloproteinase (MMP) gene expression (Gawaz et al. 2000). Platelets are activated by OxLDL, mediated by CD36 and NOX2 (Magwenzi et al. 2015). This induces the formation of platelet-monocyte aggregates, which in turn induces phenotypic changes in monocytes, promoting monocyte extravasation and enhancing foam cell formation (Badrnya et al. 2014).

Platelets Are the Central Mediators of the Progression from Ruptured Atherosclerotic Plaque to Subsequent Thrombus Formation

ACS is largely a stochastic event, and it is therefore particularly difficult to make physiological assessments immediately prior to the onset of ACS to determine possible triggers. However, various different triggers have been described, such as physical activity, emotional stress and infections. It has been suggested that the underlying mechanisms of these triggers may include autonomic activation, vasoconstriction and systemic inflammation, which are all mediated by platelets and point to possible triggers of platelet activation (Boyle et al. 2003). Vulnerable plaques that are prone to rupture are associated with a thin fibrous cap, high leukocyte content and a large necrotic core (Silvestre-Roig et al. 2014). Apoptosis of vascular smooth muscle cells (VSMCs) in the fibrous cap destabilises the plaque and is promoted by macrophage release of tumour necrosis factor (TNF)-α (Boyle et al. 2003) and MMP-mediated degradation of collagen within the fibrous cap (Silvestre-Roig et al. 2014). In addition, CD40L, which is derived from T cells and platelets, also promotes macrophage production of collagen-degrading MMPs (Libby 2013). Although platelets may play a role in these processes (Seizer and May 2013; Libby 2013; Thomas and Storey 2015b), direct evidence for a role of platelets in plaque

Table 1 Summary of major platelet-derived mediators of inflammation (adapted from Thomas and Storey 2015b)

Mediator	Class	Source	Interactions	Major role
P-selectin (CD62P)	Adhesion molecule	Surface expressed Released from α-granules	PSGL-1 on leukocytes	Mediates formation of platelet-leukocyte aggregates Cross-links leukocytes, platelets and endothelium
CD40L	Member of TNF family	Surface expressed Released from α-granules	CD40 on T cells, B cells, monocytes and dendritic cells	Promotes leukocyte adhesion to the endothelium Links innate and adaptive immune responses
Platelet factor 4 (CXCL4)	CXC chemokine	Released from α-granules	CXCR3 on leukocytes	Induces leukocyte pro-inflammatory functions, such as cytokine release, production of reactive oxygen species, phagocytosis and chemotaxis
RANTES (CCL5)	CC chemokine	Released from α-granules	CCR1, CCR3 and CCR5 on monocytes, macrophages and T cells	Promotes monocyte, macrophage and T cell chemotaxis and endothelial recruitment
IL-1	Cytokine	Released from α-granules	Multiple effects on leukocytes and endothelium	Central mediator of pro-inflammatory vascular responses and initiator of the cytokine cascade

rupture is limited. However, it is clear that platelets have a central role in mediating a prothrombotic state, which appears to be the key requirement for progression of plaque rupture or erosion towards thrombus formation and subsequent ACS.

Summary of Platelets in the Pathogenesis of Acute Coronary Syndromes

Platelet adhesion to activated endothelium and release of PF4 and RANTES promotes monocyte recruitment to atherosclerotic plaques. Following plaque rupture or erosion, platelets are activated in response to subendothelial components and play a central role in developing a thrombus within the coronary artery.

Platelet Adhesion in Acute Coronary Syndromes

Following atherosclerotic plaque rupture or erosion, circulating blood is exposed to the highly prothrombotic necrotic core of the plaque and subendothelial components, such as VWF, collagen, fibronectin and laminin (Falk et al. 2013). Exposed macrophages express tissue factor, which is a potent stimulus for thrombin generation and activation of the extrinsic coagulation cascade. Platelet interactions with mononuclear phagocytes further upregulates tissue factor expression (Lindmark et al. 2000), thus increasing thrombin generation. Atherosclerotic plaques also narrow the arterial lumen, thereby inducing haemodynamic disturbances and increasing shear stress (Crea and Liuzzo 2013). Under these conditions, platelet adhesion to exposed subendothelial components is critically dependent upon adhesion to VWF and collagen.

Initial Adhesion to the Vessel Wall Is Mediated by the GPIb-V-IX Complex

The initial adhesion of platelets to exposed subendothelial components is primarily mediated by the platelet GPIb-V-IX complex, which binds to exposed and immobilised VWF (Delaney et al. 2012). Downstream signalling, mediated by Src family kinases (Fig. 3), results in activation of $\alpha_{IIb}\beta_3$, which causes platelet aggregation (Delaney et al. 2012). VWF-mediated pulling of the GPIb unit induces the unfolding of a mechanosensitive domain, which induces platelet activation in response to high levels of sheer stress (Zhang et al. 2015). Platelet GPIb also adheres to VWF that has bound to immobilised collagen or fibrinogen, which allows platelets to bind to collagen and fibrinogen under high shear stress (Crúz et al. 2013). In addition, GPIb-V-IX signalling can be initiated by a direct action of thrombin on the GPIb-V-IX complex, which is synergistic with thrombin's activation of protease-activated receptor (PAR) 1 (Estevez et al. 2014).

A novel GPIb antagonist, anfibatide, has been shown to inhibit platelet aggregation related to VWF in a phase I study, without affecting ADP-, thrombin receptor-activated peptide (TRAP)- or collagen-mediated aggregation (Lei et al. 2014). This potently inhibited platelet adhesion, aggregation and thrombus formation ex vivo under both low and high shear stress (Lei et al. 2014). In addition, anfibatide inhibited thrombus formation in animal models of arterial injury without any apparent adverse effects on haemostasis (Lei et al. 2014). This therefore demonstrates an important role of GPIb in arterial thrombosis and shows therapeutic potential for GPIb inhibitors in ACS. Interestingly, in contradiction to the majority of other platelet mechanisms, GPIb appears to negatively regulate leukocyte trafficking and activation (Kaplan et al. 2015). Correspondingly, absence of GPIb-V-IX augments systemic inflammation in animal models (Corken et al. 2014), which may be an important consideration for clinical applications of GPIb inhibitors.

α2β1 Mediates Early Adhesion to Collagen

The integrin $\alpha_2\beta_1$ is present in a low-affinity state on resting platelets. Following initial adhesion, interaction of platelet GPIb with VWF mediates activation of $\alpha_2\beta_1$ (Crúz et al. 2005), which facilitates platelet adhesion and initiates rolling. Even in its inactive form, $\alpha_2\beta_1$ contributes towards adhesion by binding to collagen, although inside-out signalling changes its conformational shape, thereby increasing its affinity for collagen (Wang et al. 2003; Lecut et al. 2004). $\alpha_2\beta_1$ then acts synergistically with GPVI to reinforce collagen-mediated platelet activation (Jarvis et al. 2002; Kuijpers et al. 2003). $\alpha_2\beta_1$ deficiency in mice does not affect the bleeding time, and $\alpha_2\beta_1$-deficient platelets can still aggregate and adhere, although this is delayed, demonstrating that $\alpha_2\beta_1$ is not essential in the adhesion process (Nieswandt et al. 2001). However, in the absence of both $\alpha_2\beta_1$ and GPVI, platelet adhesion to collagen under flow is completely abolished (Sarratt 2005).

Firm Adhesion and Platelet Activation Is Mediated by GPVI

In distinction to many platelet receptors that are G-coupled protein receptors, GPVI belongs to the immunoglobulin superfamily (Clemetson et al. 1999), and its downstream signalling pathways involve Src family kinases. GPVI is

thought to be exclusive to platelets and megakaryocytes, and it has been estimated that there are approximately 9600 copies per platelet (Burkhart et al. 2012). GPVI is the major receptor for collagen, but is also activated by other endogenous ligands, including laminin (Inoue et al. 2006) and adiponectin (Riba et al. 2008), and the exogenous ligand CRP (Knight et al. 1999). GPVI also potentiates platelet activation in response to thrombin by mechanisms that are independent of Src kinases and Syk (Hughan et al. 2007) (Fig. 3). Platelets that lack either GPVI or a functional FcR-γ-chain display severely impaired thrombus formation at high shear stress (Best et al. 2003), suggesting that it is likely to have an important role during the high shear conditions of ACS. GPVI is present as a monomer on the resting platelet membrane and becomes a dimer on platelet activation, thereby increasing its affinity for collagen (Loyau et al. 2012). There is an increase in levels of the GPVI dimer in response to ADP and VWF, and the levels of GPVI dimers correlate with P-selectin expression (Loyau et al. 2012). It has recently been demonstrated that GPVI is also a receptor for fibrin (Alshehri et al. 2015). Binding of GPVI to fibrin promotes thrombin generation and thus amplifies platelet activation (Mammadova-Bach et al. 2015), which demonstrates an important role of GPVI in thrombus growth and stability. This explains why deficiency of GPVI increases time to occlusion following arterial injury as well as inhibiting initiation of thrombus formation in animal models (Alshehri et al. 2015). Inhibition of GPVI also decreases platelet responses to ADP, thrombin and epinephrine by approximately 20 % (Boylan et al. 2006), which suggests that GPVI is involved in amplifying platelet responses to these agonists.

Patients who are deficient in GPVI display impaired collagen-induced aggregation and adhesion and have a variable defect in haemostasis, which can be mild or severe (Grüner et al. 2005; Li et al. 2007). Consistent with this, patients with coronary artery disease with polymorphisms of GPVI also display a reduction in platelet reactivity to collagen as determined by the PFA-100 platelet assay (Lepäntalo et al. 2006). Mice with a germline knockout of GPVI and anti-GPVI antibody-treated mice display greatly reduced adhesion and aggregation to collagen and reduced thrombus formation at the site of arterial injury, at the expense of a moderate increase in bleeding time (Grüner et al. 2005; Li et al. 2007; Ungerer et al. 2011; Goebel et al. 2013). In animal models, soluble GPVI dimer inhibits platelet adhesion and aggregation to sites of vascular injury (Massberg 2003). Phase I studies have also shown that the same soluble dimeric GPVI-Fc fusion protein, Revacept, inhibits collagen-induced platelet aggregate formation in humans ex vivo (Ungerer et al. 2011). This approach also appears to improve cerebral infarct size and functional outcomes in animal models of stroke (Goebel et al. 2013) but has not yet been tested in ACS patients. Interestingly, in silico approaches have identified that losartan, a commonly used antihypertensive, may also inhibit platelet GPVI, which was confirmed by its inhibitory effects on collagen-induced platelet aggregation (Taylor et al. 2014). Kistomin, which is a snake venom metalloproteinase, has also been identified to have anti-GPVI effects as it cleaves both GPIb and GPVI, which inhibits VWF and collagen-induced platelet adhesion and aggregation, respectively (Hsu et al. 2008).

GPVI appears to also have an important role in maintaining vascular integrity during inflammation, whereas G protein-coupled receptors, such as the ADP receptors and TPα, appear to be dispensable in this process in animal models (Boulaftali et al. 2013). However, this role of GPVI appears complex and in some respects paradoxical. Platelet GPVI appears to enhance injury of the vessel wall caused by neutrophils, but also supports subsequent platelet adhesion, promoting repair of neutrophil-induced vascular breaches in animal models (Gros et al. 2015).

Summary of Platelet Adhesion

Under arterial shear stress, platelet adhesion is critically dependent on VWF and collagen. Initial adhesion and activation are mediated by GPIb-V-IX binding to VWF. This activates $\alpha_2\beta_1$, which facilitates platelet adhesion and initiates rolling. Firm adhesion is then mediated by GPVI, which also activates platelets. In addition, emerging evidence suggests an important role of GPVI as a receptor for fibrin.

Platelet Activation and Aggregation in Acute Coronary Syndromes

Following initial adhesion to the endothelium, platelets become activated by a number of specific intracellular signalling pathways, resulting in activation of the $\alpha_{IIb}\beta_3$ receptor on the surface of platelets (Fig. 2). Fibrinogen then cross-links activated $\alpha_{IIb}\beta_3$ receptors on adjacent platelets, which causes platelet aggregation. There are a number of mechanisms by which the platelet activation is amplified, involving the release of dense granules and interactions with leukocytes and the coagulation system.

GPIb and GPVI Initiate Platelet Activation During Acute Coronary Syndromes

Following plaque rupture, platelet adhesion to collagen is a potent stimulus for platelet activation. Activation of GPVI by collagen initiates platelet activation and induces

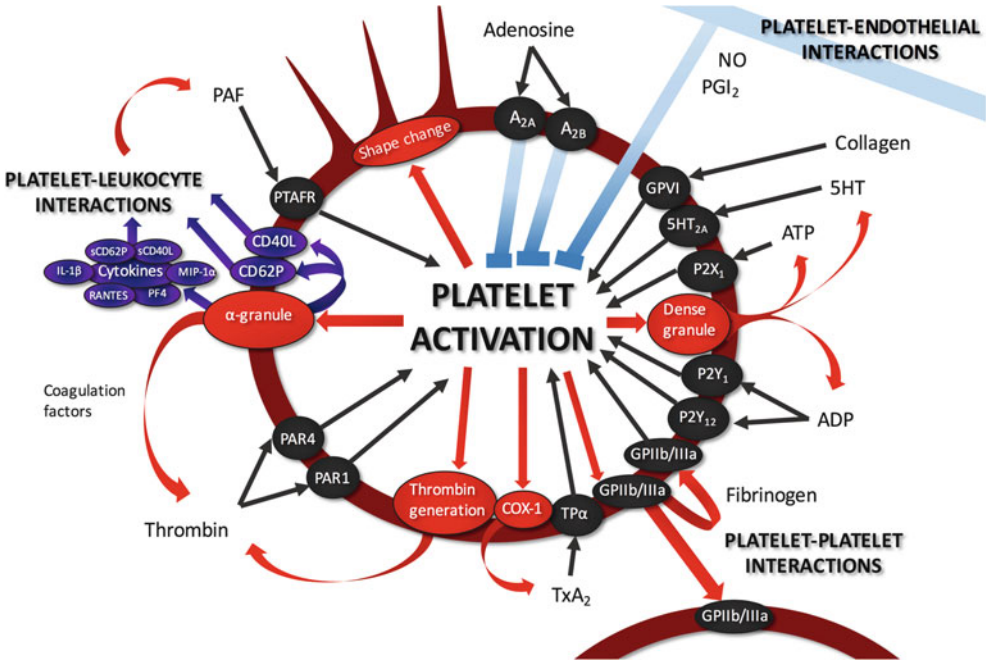

Fig. 2 Platelets are activated by multiple agonists, which act on surface receptors, leading to platelet activation, secretion of granules and generation of thrombin and thromboxane A_2

conformational changes in $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ that increase their affinity for adhering to collagen and other platelets, respectively (Lecut et al. 2004). VWF and soluble agonists induce dimerisation of GPVI and thereby increase its affinity for collagen, further potentiating platelet activation (Loyau et al. 2012). The GPVI receptor is upregulated in response to pathological shear and by components of the coagulation cascade, particularly factor Xa (Al-Tamimi et al. 2011, 2012; Naitoh et al. 2015). The receptor is shed upon platelet activation, mediated by the ADAM family of metalloproteinases, which also mediate the shedding of many other platelet membrane receptors (Bender et al. 2010). Surface expression of the collagen receptor GPVI is upregulated during ACS and correlates with expression of platelet P-selectin (Bigalke et al. 2006) suggesting that it may be a marker of platelet activation.

Platelet Activation Leads to αIIbβ3 Activation and Platelet Aggregation

$\alpha_{IIb}\beta_3$ is an adhesion receptor that belongs to the integrin superfamily and is a heterodimer composed of an α (α_{IIb}) and a β (β_3) transmembrane subunit (Coller and Shattil 2008). In the resting state, there are approximately 40,000–80,000 complexes present on the platelet membrane, and $\alpha_{IIb}\beta_3$ is only able to weakly bind fibrinogen, which is sufficient to allow uptake of fibrinogen into α-granules (Coller and Shattil 2008). The number of $\alpha_{IIb}\beta_3$ receptors on the surface membrane rapidly increases following platelet activation, which also induces conformational changes in the receptor allowing it to bind fibrinogen, VWF and fibronectin (Coller and Shattil 2008). This inside-out signalling has been described as the final common pathway of platelet activation as it is downstream of all other platelet activation signalling pathways (Coller and Shattil 2008). Fibrinogen molecules have two binding sites for $\alpha_{IIb}\beta_3$ and act as bivalent ligands, forming cross-links between activated $\alpha_{IIb}\beta_3$ receptors on different platelets, resulting in platelet aggregation (Coller and Shattil 2008). In addition, platelet $\alpha_{IIb}\beta_3$ receptors are activated by ligand binding, resulting in outside-in signalling, which is mediated by Src family kinases (Fig. 3) and causes platelet activation (Coller and Shattil 2008).

Intravenous platelet $\alpha_{IIb}\beta_3$ inhibitors are potent inhibitors of platelet aggregation and have been used for the treatment of ACS (Grove et al. 2015). However, platelet inhibition during ACS has increasingly focused on the use of platelet $P2Y_{12}$ inhibitors (Roffi et al. 2016). As $\alpha_{IIb}\beta_3$ antagonists increase the risk of major bleeding, their use is now largely limited to high-risk situations during PCI such as primary PCI for STEMI or as a "bailout" therapy for procedure-related thrombus formation (Grove et al. 2015).

Platelet Release of ADP and Subsequent Activation of Platelet $P2Y_{12}$ Receptors Is a Central Amplification Process

Platelet response to ADP is mediated by two ADP receptors, namely, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is coupled to G_q (Fig. 3) and initiates calcium mobilisation, platelet shape change and transient platelet aggregation in response to ADP (Gachet

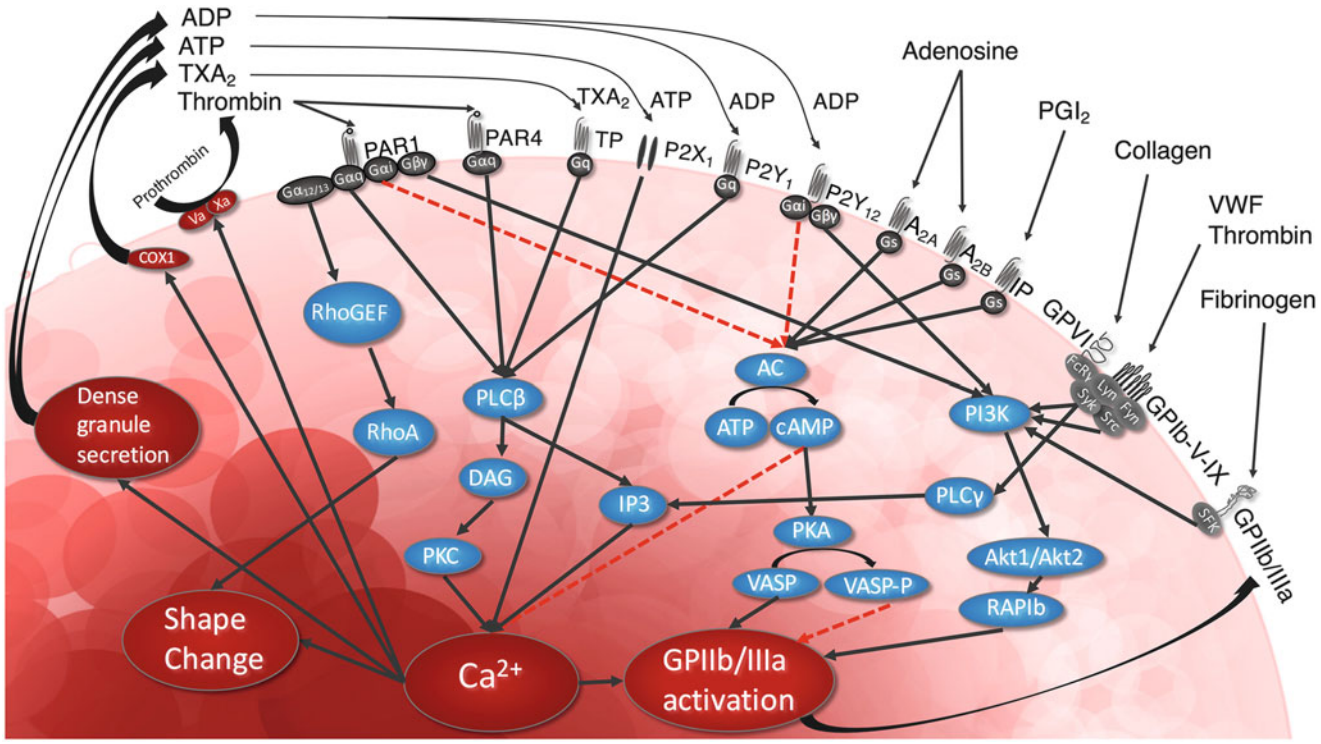

Fig. 3 Intracellular signalling following platelet activation

2012). Few attempts have been made to target $P2Y_1$ receptors pharmacologically since they are expressed in many tissues throughout the human body, including the heart, blood cells, neural tissues and other organs, making off-target effects very likely (Gachet 2012). Platelet $P2Y_{12}$ receptors are coupled to G_i and inhibit adenylyl cyclase activity, thereby decreasing levels of cyclic adenosine monophosphate (cAMP) (Gachet 2012). $P2Y_{12}$ also activates the PI3K signalling pathway, which mediates robust platelet aggregation (Cosemans et al. 2006). Platelet activation in response to ADP triggers release of platelet dense granules, which also contain ADP. The released ADP has autocrine effects that amplify the response of the activated platelet and paracrine effects that stimulate other platelets (Gachet 2012). Amplification of platelet activation by the $P2Y_{12}$ receptor has a central role in amplifying the response of platelets to other agonists (Storey et al. 2000). In addition, $P2Y_{12}$ receptors are expressed in few cell types other than platelets (Gachet 2012), which makes them an attractive target for antiplatelet pharmacotherapy. Many successful platelet $P2Y_{12}$ inhibitors have been developed and now rank amongst some of the most commonly used medications worldwide (Storey 2011). Platelet $P2Y_{12}$ inhibitors are capable of inhibiting the response of platelets to ADP and multiple other agonists, as they inhibit a central amplification pathway (Storey et al. 2000). The potent $P2Y_{12}$ inhibitors prasugrel and ticagrelor are currently recommended as first-line treatments for patients with ACS, in addition to aspirin (Roffi et al. 2016).

The Platelet Agonist Thromboxane A_2 Is Synthesised upon Platelet Activation

Arachidonic acid is formed by phospholipase A_2, which hydrolyses membrane phospholipids. Arachidonic acid is then rapidly converted by cyclooxygenase (COX-1) in platelets into prostaglandin G_2, which is then converted into prostaglandin H_2 (PGH_2) by peroxidase (Patrono et al. 2005). Thromboxane synthase converts PGH_2 into thromboxane A_2 (TxA_2), which can diffuse out through the platelet membrane to then act upon platelet TP receptors to amplify platelet activation (Patrono et al. 2005). Aspirin inhibits COX-1, thereby reducing the synthesis of TxA_2, which strongly inhibits arachidonic acid-induced platelet aggregation and moderately inhibits collagen-induced platelet aggregation (Storey et al. 2000). In citrate-anticoagulated platelet-rich plasma, aspirin also inhibits ADP- and adrenaline-induced platelet aggregation (Storey et al. 2000). However, these effects are not seen at physiological levels of calcium, which suggests that the arachidonic acid pathway has a limited role in platelet activation under physiological conditions (Heptinstall and Mulley 1977).

The antiplatelet properties of low-dose aspirin are of great benefit in patients with ACS (Collaboration 2002). Since the successful introduction of aspirin, it is now used routinely in combination with platelet $P2Y_{12}$ inhibitors (Storey 2011). Further studies are therefore needed to determine optimal usage of aspirin when it is used on a background of potent $P2Y_{12}$ inhibition (Thomas and Storey 2014a). Specific thromboxane receptor (TP) inhibitors have also been developed. Ridogrel, a combined TXA_2 synthase and TP receptor inhibitor, is the only such drug to have been tested in patients with ACS. However, ridogrel did not improve indices of reperfusion in patients with STEMI undergoing thrombolysis compared to aspirin and further development of TP receptor inhibitors has since ceased (investigators 1994).

Amplification of Platelet Activation by Protease-Activated Receptors

Platelets express two different receptors for thrombin, namely, PAR1 and PAR4 (Leger et al. 2006). Thrombin is the most potent activator of platelets and is thought to exert the majority of its effects through PAR1 (Leger et al. 2006). PARs have an unusual mechanism of action, in that a cryptic ligand within the receptor is unmasked by proteolytic cleavage and the tethered ligand then interacts with the receptor moieties (Leger et al. 2006). PAR1 activates $G_{12/13}$, resulting in marked shape change and formation of pseudopodia, as well as dense granule release (Fig. 3). PAR1 is also linked to G_q, which increases intracellular calcium and activates αIIbβ3 (Leger et al. 2006). Whilst PAR1 has a high affinity for thrombin and is activated at subnanomolar concentrations of thrombin, PAR4 has a lower affinity and is cleaved more slowly (Leger et al. 2006). Despite this, PAR4 is able to mediate robust increases in intracellular calcium and platelet aggregation, mediated by G_q (Leger et al. 2006).

The PAR1 receptor antagonist vorapaxar initially showed great promise in early-phase studies as it appeared to reduce platelet aggregation without adversely affecting haemostasis. However, in patients with ACS, vorapaxar increased the risk of bleeding and only showed a trend for cardiovascular benefits (Tricoci et al. 2012). Vorapaxar did significantly reduce the incidence of adverse cardiovascular events in patients with stable coronary artery disease with a prior history of MI and has recently been licensed for this indication (Morrow et al. 2012).

Summary of Platelet Activation

Following adhesion to the endothelium, platelets become activated in response to VWF and collagen in addition to a number of soluble mediators, which activates specific intracellular signalling pathways. These signalling pathways induce calcium mobilisation, shape change and activation of $\alpha_{IIb}\beta_3$, which is cross-linked with $\alpha_{IIb}\beta_3$ on adjacent platelets by fibrinogen. This results in platelet aggregation.

Platelet-Leukocyte Interactions

There are a number of mechanisms by which activated platelets interact with leukocytes and the endothelium. Activated platelets express the adhesion molecule P-selectin on their surface membrane, which interacts with its corresponding ligand, P-selectin glycoprotein ligand (PSGL)-1, on monocytes, neutrophils and eosinophils (Thomas and Storey 2015b). This mediates the formation of platelet-leukocyte aggregates, and circulating levels of platelet-monocyte aggregates have been shown to be increased in patients with ACS (Michelson et al. 2001). Increased platelet expression of P-selectin despite antiplatelet therapy has been shown to be associated with a higher incidence of adverse cardiovascular events in patients with ACS (Thomas et al. 2014). Inhibition of P-selectin reduces the formation of platelet-monocyte aggregates in vivo (Tardif et al. 2013). A novel specific antibody against P-selectin, inclacumab, lowers levels of soluble P-selectin and shows trends towards reduced myocardial damage in patients with ACS, presumably mediated by reducing platelet-leukocyte interactions (Tardif et al. 2013).

Platelets also express CD40L on their surface membrane when activated. CD40L has a similar structure to TNFα and has effects that are similar (Thomas and Storey 2015b). Platelet CD40L acts on monocyte CD40 to increase monocyte expression of tissue factor, which exerts prothrombotic effects by activating the extrinsic coagulation cascade (Thomas and Storey 2015b). CD40L is also an important mediator of adaptive immune responses and the regulation of T cells in particular (Thomas and Storey 2015b). Patients with ACS have an increased plasma level of soluble CD40L, which is thought to derive from platelets since they are the main source (Blanchet et al. 2014). Reflecting high levels of platelet activation, elevated levels of CD40L are associated with an increased risk of mortality in patients with ACS (Blanchet et al. 2014).

Platelets Interact with Monocytes, Promoting the Release of Pro-inflammatory Cytokines

As well as containing many mediators of coagulation, platelet α-granules contain a large number of inflammatory mediators that serve no role in haemostasis (Thomas and Storey 2015b). Upon activation, platelets release cytokines

such as interleukin (IL)-1β, chemokine (C-X-C motif) ligand (CXCL) 1, PF4, CXCL5, CXCL7, IL-8, CXCL12, macrophage inflammatory protein (MIP)-1α and RANTES (Thomas and Storey 2015b). PF4, RANTES and MIP1α are important promoters of monocyte chemotaxis and adhesion to the endothelium and have been directly implicated in atherogenesis (Thomas and Storey 2015b). Levels of PF4, RANTES and MIP1α are increased during ACS (Blanchet et al. 2014), and high levels of RANTES are associated with more rapid subsequent progression of atherosclerotic plaques (Blanchet et al. 2014). Increased levels of RANTES and MIP1α are also both associated with a threefold increase in risk of mortality (de Jager et al. 2012; Blanchet et al. 2014).

The formation of platelet-monocyte aggregates increases monocyte release of pro-inflammatory cytokines, such as TNFα, MCP-1 and IL-8 (Thomas and Storey 2015b), which represents a mechanism by which platelets amplify systemic inflammation during ACS. Patients with ACS who have high levels of baseline inflammatory markers have an increased risk of subsequent adverse cardiovascular events and mortality (Thomas and Storey 2015c). Platelet $P2Y_{12}$ inhibitors have been shown to reduce systemic inflammation and also lower levels of inflammatory markers during ACS, which may contribute to their clinical benefit (Thomas and Storey 2015c; Thomas et al. 2015).

Platelets Interact with Neutrophils, Resulting in the Formation of Neutrophil Extracellular Traps

Neutrophil counts are often raised in patients with ACS, and there is evidence for a rapid burst of neutrophil activation and release of myeloperoxidase (MPO) in the first few hours of ACS, which is associated with platelet-neutrophil aggregate formation (Maugeri et al. 2012). Platelets interact with neutrophils to promote the formation of neutrophil extracellular traps (NETs), mediated by the platelet TLR4 receptor (Clark et al. 2007). NETs are normally extruded by neutrophils as a means of ensnaring bacteria and consist of cytoplasmic proteins and nuclear contents (Thomas and Storey 2015b). Whilst this may be beneficial to aid the clearance of bacteria, it is becoming increasingly clear that these NETs also play a significant role in the pathological inflammation and thrombosis (Thomas and Storey 2015b). In patients with STEMI, it has recently been demonstrated that a significant proportion of coronary thrombi are actually made up of NETs in close relation to platelets (Maugeri et al. 2014). Furthermore, it has also been shown that coronary NET burden is a predictor of subsequent infarct size (Mangold et al. 2015). Deoxyribonuclease hydrolyzes the DNA scaffold of NETs and has been shown to accelerate the lysis of coronary thrombi ex vivo, suggesting that NETs could be a pharmacological target for the treatment of ACS (Mangold et al. 2015).

Platelets Release Microparticles, Which Are Important Mediators of Inflammation and Coagulation and also Contain MicroRNA

Platelets release small membrane vesicles upon activation, known as microparticles (Biasucci et al. 2012). Platelets are a major source of microparticles, but other cell types, including endothelial cells, leukocytes and VSMCs, all release different types of microparticles as well (Vajen et al. 2015). Microparticles mediate pro-inflammatory and procoagulant interactions between many different cell types and have effects that include increasing the thrombogenicity of platelets and promoting monocyte interactions with the endothelium. Levels of platelet-derived microparticles are increased during ACS (Biasucci et al. 2012), suggesting their potential use as a biomarker or as a pharmacological target in patients with ACS.

miRNA are small non-coding RNA, which function as translational repressors to modulate gene expression (Willeit et al. 2013). They have a diverse range of effects across a range of cell types and have been shown to have important roles in modulating endothelial cell function and inflammatory responses. Interestingly, platelet microparticles appear to be a predominant source of miRNA in the plasma (Willeit et al. 2013). miR-126 and miR-223 are particularly abundant within platelets, and their release can be inhibited by platelet $P2Y_{12}$ inhibitors and aspirin (Willeit et al. 2013). In patients with ACS, levels of miR-126 and miR-223 both correlate with indices of platelet reactivity, which suggests that they may be useful as a novel marker of platelet activation in plasma (Mayr et al. 2014).

Platelets Mediate the Formation of a Stable Thrombus

In their resting state, platelets have an asymmetrical phospholipid membrane that is maintained by translocase. Upon activation, increased levels of cytoplasmic calcium inhibit translocase and activate scramblase, which enhances the expression of negatively charged phosphatidylserine on the outer membrane. This allows factor Xa and Va to form a prothrombinase complex on the surface membrane, which converts prothrombin into thrombin (Solum 1999). The produced thrombin activates platelet PARs and also leads to fibrin formation, which binds the developing clot together. In addition, platelets synthesise large quantities of plasminogen activator inhibitor (PAI)-1

and release this from α-granules upon activation (Brogren et al. 2004). The balance between clot formation and lysis is closely regulated by the fibrinolytic system, and tissue plasminogen activator (tPA) in particular plays an important role on clot lysis. PAI-1 inhibits the fibrinolytic action of tPA and thereby increases resistance to clot lysis, particularly in arterial thrombosis (Brogren et al. 2004). High plasma concentrations of PAI-1 have been demonstrated in patients with ACS and increased levels are associated with an increase in mortality (Collet et al. 2003). Platelet-monocyte aggregate formation has also been linked with altered fibrinolytic status during ACS, which may be related to the intermediate monocyte phenotype in particular (Shantsila et al. 2012). In patients with ACS, there is a dynamic cycle between prothrombotic and prothrombolytic processes, which occur simultaneously (Falk et al. 2013). This leads to intermittent flow obstruction and distal embolisation, which may contribute towards myocardial microvascular obstruction and is more common following plaque erosion rather than rupture (Falk et al. 2013).

Summary of Platelet-Leukocyte Interactions

Activated platelets express P-selectin on their surface membranes, which interacts with PSGL-1 on leukocytes and mediates the formation of platelet-leukocyte aggregates. In addition, platelets release numerous pro-inflammatory cytokines (Table 2) from their α-granules upon activation. More recently it has also been shown that platelets interact with neutrophils, mediating the formation of NETs, which are prothrombotic and contribute to the scaffolding of the thrombus.

Table 2 Major platelet receptors and corresponding ligands

Receptor	Type	Ligand
GPIb-V-IX	Membrane receptor complex	VWF Thrombin
$\alpha_2\beta_1$	Membrane glycoprotein	Collagen
GPVI	Membrane glycoprotein	Collagen Fibrin
αIIbβ3	Integrin complex	Fibrinogen VWF
$P2Y_1$	G protein-coupled receptor	ADP
$P2Y_{12}$	G protein-coupled receptor	ADP
$P2X_1$	Ligand-gated ion channel	ATP
PAR1	G protein-coupled receptor	Thrombin
PAR4	G protein-coupled receptor	Thrombin
TPα	G protein-coupled receptor	Thrombin
$5HT_{2A}$	G protein-coupled receptor	Serotonin
A_{2A}	G protein-coupled receptor	Adenosine
A_{2B}	G protein-coupled receptor	Adenosine

Platelet Contribution Towards Microvascular Obstruction and Risk of Subsequent Restenosis and Adverse Cardiovascular Events

After atherosclerotic plaque rupture, the thrombus rapidly grows in size and may obstruct, or partially obstruct, the coronary artery, leading to myocardial ischaemia that may be fatal. Acute total occlusion of a coronary artery by thrombus usually results in STEMI, and contemporary management of this revolves around emergency primary percutaneous coronary intervention (PCI) to reopen the affected artery. The aim is to achieve this as quickly as possible, to minimise the duration of myocardial ischaemia. However, even when successful primary PCI is prompt and potent antithrombotic agents are administered, myocardial ischaemia persists in a significant proportion of patients due to microvascular obstruction further downstream of the initial obstruction (Niccoli et al. 2016). Platelets contribute towards some of the main mechanisms of microvascular obstruction, which involve ischaemia, reperfusion injury and distal embolisation (Niccoli et al. 2016). Platelet-neutrophil aggregates damage the endothelium of the microvasculature by releasing inflammatory mediators and vasoconstrictors (Niccoli et al. 2016). Additionally, platelet NOX2 is upregulated during microvascular obstruction, which exacerbates the underlying pathophysiology by promoting phospholipase A_2 activity and generating TxA_2, a potent vasoconstrictor and platelet agonist (Niccoli et al. 2013). Patients with STEMI that is complicated by microvascular obstruction have higher levels of platelet-monocyte aggregates, platelet-neutrophil aggregates and activated $\alpha_{IIb}\beta_3$ than those without microvascular obstruction (Zalewski et al. 2012). Furthermore, levels of platelet-derived microparticles have been shown to be increased at the site of coronary plaque rupture, and higher levels are associated with microvascular obstruction in patients with STEMI (Porto et al. 2012). In animal models, neutrophils release NETs during myocardial ischaemia-reperfusion, which contributes to the pathophysiology of the condition (Brinkmann and Zychlinsky 2012). Interestingly, deoxyribonuclease is able to degrade the backbone of NETs and is effective at reducing the ischaemic region and infarct size in this situation (Ge et al. 2015).

Following atherosclerotic plaque rupture, there is evidence to suggest that platelets play an important role in progression of atherosclerotic plaques. By forming a thrombus over the site of atherosclerotic plaque rupture, platelets may contribute towards atherosclerosis by releasing pro-inflammatory mediators and enhancing the recruitment of monocytes (Libby 2000). In an animal model, $P2Y_{12}$-deficient mice displayed a significant reduction in thrombus and neointima formation following arterial injury (Evans et al. 2009). Further bone marrow transplantation experiments suggested that the reduction in neointima formation was primarily due to platelet $P2Y_{12}$ deficiency rather than VSMC $P2Y_{12}$ deficiency (Evans

et al. 2009). This demonstrates a mechanism by which platelet $P2Y_{12}$ inhibitors may reduce restenosis following PCI in patients with ACS. In addition, platelet activation and secretion of dense granules releases ATP, which may act on VSMC P2X receptors, thereby promoting VSMC proliferation and migration (Erlinge 1998).

Treatment with a $P2Y_{12}$ inhibitor, in addition to aspirin, is recommended for at least a year after ACS (Roffi et al. 2016). The more recent and potent $P2Y_{12}$ inhibitors ticagrelor and prasugrel are recommended as first-line treatments for patients with ACS (Roffi et al. 2016). It has become clear that approximately a third of patients do not display adequate inhibition of platelet reactivity whilst on treatment with the older $P2Y_{12}$ inhibitor clopidogrel (Aradi et al. 2015). The reasons for this poor response to clopidogrel are multifactorial and incompletely understood, but in part relate to genetic polymorphisms (Thomas and Storey 2014b) and drug-drug interactions amongst other factors (Thomas and Storey 2011). High platelet reactivity to ADP despite treatment with clopidogrel is associated with an increased risk of subsequent adverse cardiovascular events in ACS patients (Aradi et al. 2015). Studies have investigated whether this poor response to clopidogrel can be ameliorated by increasing the dose or changing to prasugrel, but unfortunately have not shown any significant benefit so far (Collet et al. 2015). However, even with the use of potent platelet $P2Y_{12}$ inhibitors, patients with ACS continue to have an increased risk of mortality, demonstrating the need for novel developments in antithrombotic strategies.

Summary

Platelets are central to all stages of the pathophysiology of ACS (Table 3). Platelets release many pro-inflammatory mediators, such as PF4, RANTES and sCD40L, which have been implicated in the progression of advanced atherosclerotic plaques. Following plaque rupture or erosion, platelets bind to exposed VWF and collagen, mediated by GPIb, $\alpha_2\beta_1$ and GPVI. Initial platelet activation is then amplified by platelet release of soluble agonists, such as ADP, TxA_2 and thrombin, which stimulates further platelet activation in an autocrine and paracrine manner. Fibrinogen cross-links activated platelet $\alpha_{IIb}\beta_3$ receptors, causing platelet aggregation, and the procoagulant activities of platelets drive the development of a stable fibrin-bound clot. Emerging evidence also suggests that platelet-leukocyte interactions have an important role in clot formation and microvascular obstruction. Aspirin and $P2Y_{12}$ inhibitors have made a tremendous impact on improving outcomes from ACS, which paves the way for novel antiplatelet strategies to further benefit patients with ACS by targeting currently unexploited mechanisms.

Table 3 Summary of the major roles of platelets in the pathophysiology of ACS

Pathophysiology of ACS	Platelet involvement
Progression of advanced atherosclerotic plaque	Activated platelets promote leukocyte recruitment to the endothelium by upregulating expression of ICAM-1 and release of MCP-1 Platelet release of PF4, RANTES and CD40L promotes macrophages to infiltrate atherosclerotic plaques, change phenotype and produce collagen-degrading MMPs (Gawaz et al. 2000; Hundelshausen et al. 2005; Libby 2013) OxLDL induces the formation of platelet-monocyte aggregates, which promotes monocyte extravasation and enhances foam cell formation (Badrnya et al. 2014)
Platelets adhere to exposed subendothelial components, initiating thrombus formation	Platelet GPIb initiates platelet adhesion and activation by binding exposed VWF (Delaney et al. 2012) Platelet $\alpha_2\beta_1$ mediates adhesion by binding exposed collagen (Jarvis et al. 2002; Kuijpers et al. 2003) Platelet GPVI acts synergistically with α2β1 to facilitate adhesion and potently activates platelets in response to collagen (Jarvis et al. 2002; Kuijpers et al. 2003)
Activated platelets aggregate at the site of plaque rupture	Fibrinogen cross-links activated $\alpha_{IIb}\beta_3$ receptors on adjacent platelets, causing aggregation (Coller and Shattil 2008)
Platelet activation is amplified in an autocrine and paracrine manner	Platelets release ADP from their dense granules, which activates platelet $P2Y_{12}$ ADP receptors, causing further activation (Storey et al. 2000) Platelets synthesise thromboxane A_2, which acts on platelet TP receptors (Patrono et al. 2005)
Stable thrombus occludes, or partially occludes, the coronary lumen	Platelets activation induces the formation of a prothrombinase complex on their surface membrane, which converts prothrombin into thrombin (Solum 1999) Thrombin activates platelet PARs and also promotes fibrin generation, which consolidates clot formation (Leger et al. 2006)
Inflammation induced by ACS is mediated by platelet-leukocyte interactions	Platelet-monocyte aggregate formation promotes monocyte release of pro-inflammatory cytokines (Thomas and Storey 2015b) Platelet-neutrophil interactions induces NET formation and may play an important role in the pathophysiology of microvascular obstruction

Take Home Messages

- Platelets play a role in the development of atherosclerosis and in the formation of a thrombus after atherosclerotic plaque rupture.
- If a thrombus occludes the coronary artery, this may lead to an acute coronary syndrome.
- Inhibition of platelet-specific receptors has proved to be an exceptionally successful approach in the treatment of acute coronary syndromes.
- Although many receptor antagonists have been investigated, several receptors have still not been targeted in patients with acute coronary syndromes, which offer the potential for exciting, novel antiplatelet strategies.

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Platelets in Acute Ischemic Stroke

Guido Stoll, Christoph Kleinschnitz, and Bernhard Nieswandt

Abstract

Ischemic stroke and myocardial infarction are the major causes of death and disability worldwide. Rapid restoration of blood flow by pharmacological thrombolysis and/or mechanical thrombectomy is the mainstay of acute stroke treatment, but does not guarantee a favorable outcome. Reperfusion injury denotes the acute, paradoxically harmful aspect of blood flow return in the ischemic brain which involves platelet activation and, surprisingly, immune cell recruitment. In experimental stroke, glycoprotein (GP)Ibα facilitated tethering of platelets to the postischemic brain endothelium by binding to von Willebrand factor (VWF), while firm adhesion and platelet activation were mediated by GPVI signaling. Accordingly, blocking of platelet GPIbα or GPVI, as well as reducing circulating VWF, dramatically improved stroke outcome by protecting the microvasculature during reperfusion and in addition accelerated recanalization during thrombolysis. Despite interfering with platelet function, no bleeding complications occurred, in contrast to devastating intracranial hemorrhages observed after blocking platelet aggregation via $\alpha_{IIb}\beta_3$. It will be essential to further dissect pathological platelet functions and activation pathways involved in reperfusion injury from those indispensable as gatekeepers of hemostasis in the stroke-injured brain. The pathophysiology of acute stroke is even more complex since it involves concerted detrimental actions of platelets and T-cells, referred to as "thrombo-inflammation," which await further elucidation.

Introduction

Stroke is a leading cause of death worldwide and significantly contributes to permanent disability in the aging world population Global Burden of Disease Study 2013 collaborators 2015). Cerebral ischemia accounts for roughly two thirds of strokes, while the remaining cases are caused by primary intracerebral hemorrhages (ICH) (Krishnamurthi et al. 2013) which are not covered in this chapter. Atrial fibrillation (AF) and high-grade internal carotid artery (ICA) stenosis represent the major sources of cerebral thromboembolism (Lip and Lane 2015; Stoll and Bendszus 2006). Thromboembolic occlusion of major or multiple smaller intracerebral arteries leads to impairment or cessation of the downstream blood flow (Fig. 1a, b) which triggers a plethora of consecutive pathological events in the brain leading to ischemic brain injury (Stoll et al. 1998; Dirnagl et al. 1999). For decades, prevention has been the mainstay of stroke treatment. Anticoagulation is the treatment of choice for prevention of cardiogenic thromboembolism in AF patients (Lip and Lane 2015), while thromboembolism emerging from atherosclerotic plaques of extracranial

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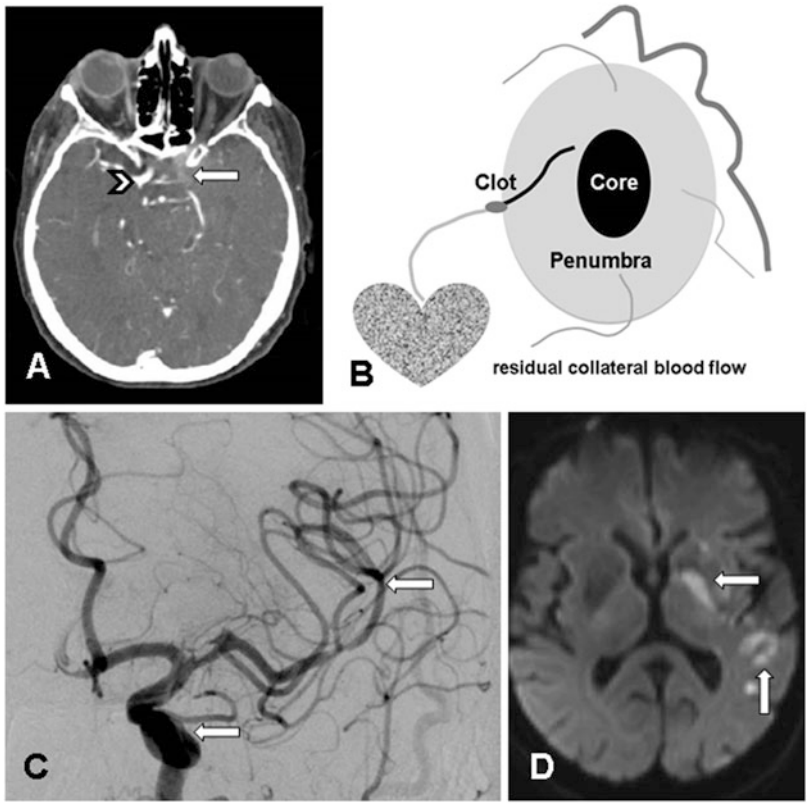

Fig. 1 Successful recanalization and favorable clinical outcome in a patient with acute internal carotid artery (ICA) occlusion. (**a**) CT angiogram on admission showing a patent distal ICA on the right side (*arrowhead*) and a missing contrast enhancement due to occlusion of the left ICA (*arrow*). Clinically, the patient exhibited hemiplegia on the right side and was aphasic. (**b**) Depicts the penumbra concept: After occlusion of a major cerebral artery (like in this patient), severe hypoxia occurs in the center of the corresponding vascular territory (core), while in the surround, called penumbra, residual blood flow is maintained by collaterals. Thereby, the penumbra contains salvageable brain tissue upon successful reperfusion. (**c**) Shows a conventional digital subtraction angiogram after successful removal of the vessel-occluding clot by mechanical thrombectomy. Note recanalization of the ICA and the branches of the MCA (*arrows* in **c**). According to a good clinical outcome with a residual hemiparesis and mild aphasia, diffusion-weighted magnetic resonance imaging, the most sensitive sequence for detection of ischemic brain lesions, showed only small embolic lesion areas, which appear white (*arrows* in **d**)

vessels is amenable to carotid endarterectomy/stenting (Stoll and Bendszus 2006) and/or conventional antiplatelet therapies (Sandercock et al. 2014). The clinical aspects of antiplatelet therapy in primary and secondary stroke prevention are discussed in chapter by Spokoysy and Albers (2017 in this volume); we here focus on the pathophysiological role of platelets in the acute phase of stroke, e.g., within 24 h of symptom onset, with emphasis on reperfusion injury of the brain. While immune cell recruitment has been thoroughly analyzed up to weeks after stroke onset (Stoll et al. 1998; Gelderblom et al. 2009) to the best of our knowledge, no similar studies on secondary recruitment of platelets and their role within the brain parenchyma in the subacute and chronic stages of infarct maturation are available. There is evidence that lesion-induced accumulation of platelets promotes survival of adult neural stem cells in the brain (Kazanis et al. 2015), and, moreover, platelets play a decisive role in wound healing (Nurden 2011). Whether platelets are involved in lesion maturation and tissue remodeling after brain ischemia, and if late outcome could be modified by antiplatelet treatment, is completely unknown and therefore not covered in this chapter. Prompt application of antiplatelet drugs in stroke patients is currently based solely on their effects on secondary prevention of recurrent thromboembolic events (Sandercock et al. 2014).

Acute Stroke Treatment: Thrombolysis and Mechanical Thrombectomy

The main goal of treatment in acute ischemic stroke is recanalization of occluded extra- and intracranial vessels (Fig. 1). Intravenous thrombolysis by recombinant tissue-type plasminogen activator (rt-PA) within 4.5 h after symptom onset introduced in 1995 was the only proven effective treatment for acute stroke (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group

1995). Broad clinical experience showed that recanalization was the strongest predictor of good clinical outcome, but it also became obvious that only a low rate of recanalization could be achieved by intravenous thrombolysis alone in proximal vessel occlusion, e.g., occlusion of the ICA or the M1 segment of the middle cerebral artery (MCA) (Bhatia et al. 2010). This prompted further clinical trials evaluating intra-arterial thrombolysis and mechanical thrombectomy using stent retriever systems which at the beginning revealed equivocal results. The recent MR CLEAN trial (Berkhemer et al. 2015) was the first to document superiority of mechanical thrombectomy over pharmacological thrombolysis alone and has changed stroke care worldwide (Grotta and Hacke 2015). Five hundred patients with acute stroke were randomized to standard thrombolysis with and without intra-arterial thrombectomy within 6 h of symptom onset. Although the reperfusion rate of 58.7 % in MR CLEAN was relatively low as compared with previous,

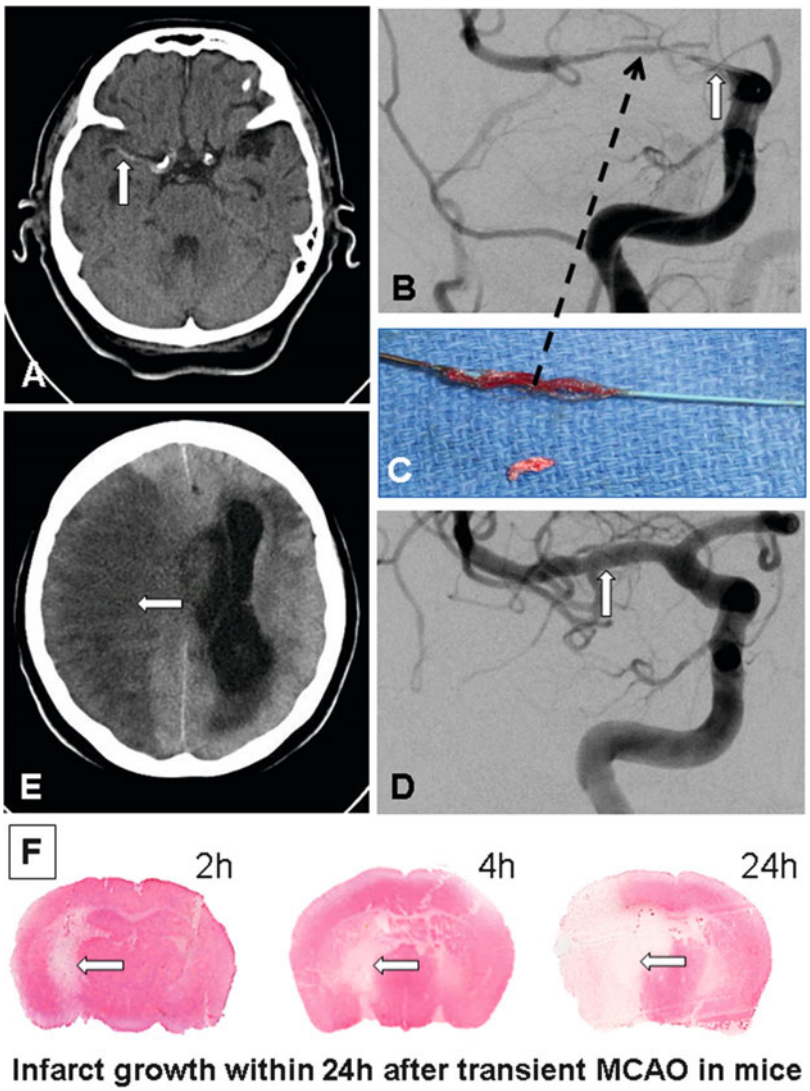

Fig. 2 Infarct development despite successful recanalization. A patient with acute left-sided hemiparesis due to occlusion of the right middle cerebral artery (MCA). The intravascular clot appears white on the native CT scan (*arrow* in **a**). A catheter device (stent retriever) is advanced through the femoral artery into the internal carotid artery and further upward to the proximal end of the intravascular clot within the MCA; the fresh clot is penetrated (*white arrow* in **b** denotes proximal site of occlusion), captured by the devise, and a stent then extended which facilitates immediate restoration of blood flow. The stent retriever system containing the former vessel-occluding clot (shown in **c**) is then removed and the patency of the MCA completely restored (*arrow* in **d** depicts the reperfused MCA segment). Despite successful recanalization, the patient unfortunately developed a large MCA infarction due to reperfusion injury (**e**). This common clinical situation can be mimicked by the transient MCA occlusion model in mice: (**f**) show infarct development in mice after 1 h of MCAO by TTC staining, an indicator for vital tissue. Dark areas show vital brain tissue, while pale areas are necrotic marked by arrows. Note that 2 h and 4 h after reperfusion, cortical areas are still spared from ischemic brain damage, but despite recanalization a full-blown MCA infarct inevitably develops within 24 h

clinically equivocal trials which were 80 % or higher, for the first time there was an absolute difference in the rate of functional independence (modified Rankin score 0–2) in favor of the intervention (32.6 % vs. 19.1 %) (Berkhemer et al. 2015). After publication of MR CLEAN, a number of concurrent clinical studies were prematurely halted after interim analyses confirming these encouraging results (Grotta and Hacke 2015). Mechanical thrombectomy increases the number of acute stroke patients with successful restoration of blood flow and favorable outcome (Fig. 1), but a considerable number of patients still develops severe neurological deficits or die, despite recanalization (Fig. 2). The number needed to treat for a good functional outcome in one patient is around four to six over all thrombectomy trials. The mechanisms underlying infarct growth despite recanalization are largely unknown, but there is increasing evidence from animal studies that platelets are critically involved.

The Concept of Reperfusion Injury After Brain Ischemia and Early Observations Regarding the Function of Platelets Herein

As discussed, revascularization therapies in stroke patients aim to rescue brain tissue at risk, the so-called ischemic penumbra (Jung et al. 2013), by restoring the patency of an occluded major supplying artery (recanalization) (Fig. 1) and the downstream capillary blood flow (reperfusion) (Soares et al. 2010). Importantly, recanalization does not necessarily facilitate reperfusion (Nour et al. 2013). The phenomenon that "blood does not flow despite recanalization" has been termed "no-reflow" phenomenon (Ames et al. 1968), and the paradoxically harmful aspect of blood flow return in transiently ischemic organs has been termed "reperfusion injury" (Hallenbeck and Dutka 1990; del Zoppo and Mabuchi 2003). In the brain, conversion of the luminal surface of the postischemic endothelium within the microvasculature from an anticoagulant to a procoagulant membrane has been proposed as one key mechanism of these harmful events (Hallenbeck and Dutka 1990). In support of this notion, Del Zoppo and colleagues could show that ^{111}In-labeled platelets are deposited in the ischemic basal ganglia early during reperfusion in a primate model of transient middle cerebral artery occlusion (tMCAO) (Del Zoppo et al. 1986). Electron microscopic examination of the microvasculature within the ischemic region further demonstrated aggregates of degranulated platelets together with fibrin and leukocytes and provided direct evidence that platelet activation occurs in the ischemic zone (Okada et al. 1994). Baboons treated with ticlopidine, a first generation P_2Y_{12}-ADP receptor inhibitor, in combination with heparin displayed a significant reduction in platelet deposition and in the number of microvascular occlusions in the ischemic basal ganglia (Del Zoppo et al. 1986). In clinical practice, platelet aggregation inhibitors, most notably acetylsalicylic acid (ASA), are routinely given to ischemic stroke patients not amenable to recanalization treatments within 48 h of symptom onset (Sandercock et al. 2014). This recommendation is based on the results of two major clinical trials involving more than 40,000 participants, in which treated patients had a moderate, but statistically significant, benefit on stroke outcome (CAST (Chinese Acute Stroke Trial) Collaborative Group 1997; International Stroke Trial Collaborative Group 1997). It is widely held, however, that the primary effect of ASA was due to reduction of early recurrent stroke rather than halting ongoing subacute lesion development in the brain (Jauch et al. 2013). A recent retrospective, case-control study in 3025 patients with first-ever ischemic stroke, however, provided class II evidence that patients with pre-stroke antiplatelet agent use were partly protected in the acute phase as they showed decreased stroke severity (Jung et al. 2015). On the other hand, treatment of stroke patients with ASA immediately after intravenous thrombolysis provided no evidence for a beneficial antithrombotic effect, but was associated with early deterioration caused by an increased rate of ICH (Zinkstok et al. 2014) (Fig. 4). Thus, although the protective role of platelet aggregation inhibitors in primary and secondary stroke prevention is well established (CAPRIE Steering Committee 1996; Bhatt et al. 2006) (see Spokoyny and Albers (2017)), the functional role of platelets during the acute stroke phase is less well established and awaits further clarification (Sandercock et al. 2014). The task is to dissect detrimental platelet effects in reperfusion injury from their essential function as gatekeepers of hemostasis in acute stroke. The tremendous progress made in our understanding of the mechanisms of platelet activation, as well as the receptors and signaling molecules involved (as described in detail in other chapters of this book), allows translational research to better define the role of platelets during cerebral ischemia. Thereby the transient middle cerebral artery occlusion model (tMCAO) of focal cerebral ischemia is widely used (Braeuninger et al. 2012). Usually a filament is inserted into the ICA of rodents and further advanced intracranially to occlude the MCA for variable time periods. In most studies the final stroke volume is assessed around 24 h after onset of ischemia. The extent of ischemic brain damage depends on the occlusion time until reperfusion is allowed by removal of the filament. It is important to note that despite reperfusion, infarcts further develop and maturate within the following 24 h. In mice a MCA occlusion time of 1 h finally leads to complete infarction of the MCA territory, but infarcts gradually grow during reperfusion as shown in Fig. 2f [for further details see Braeuninger et al. (2012)]. Thus, the tMCAO model partly mimics the clinical situation in which patients with a thrombotic MCAO undergo thrombolysis or mechanical thrombectomy and, in

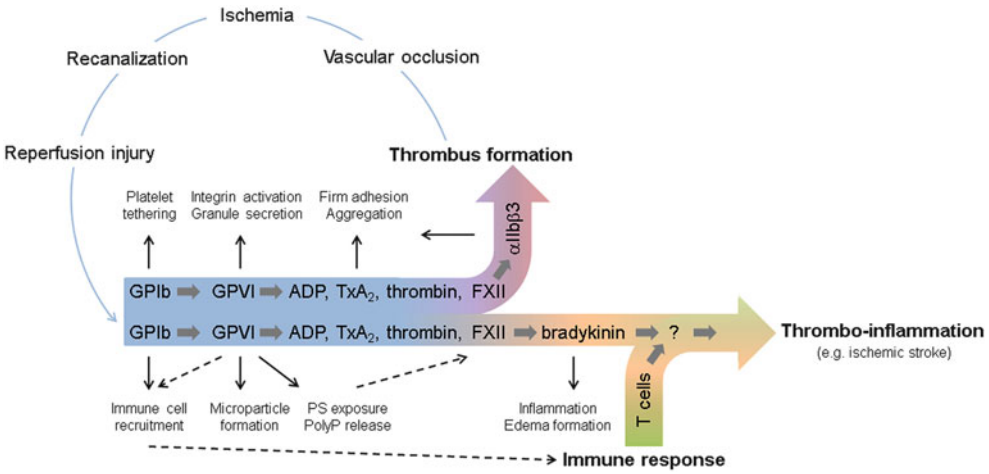

Fig. 3 Distinct but overlapping mechanisms drive thrombus formation and thrombo-inflammatory responses. GPIb mediates platelet tethering at the site of vascular injury, but also participates in immune cell recruitment during inflammatory responses. The central activating collagen receptor on the platelet surface, GPVI, supports platelet activation and triggers granule release which is essential for the secretion of ADP and TxA_2 that together with locally produced thrombin potentiate platelet activation. In parallel, strong cellular activation via GPVI culminates in the exposure of procoagulant phosphatidylserine (PS) and the release of inorganic polyphosphates (PolyP) which fuels coagulation and potently activates FXII, respectively. Additionally, GPVI signaling has been implicated in immune cell recruitment and inflammation via microparticle formation. Integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) is essential for stable thrombus formation which may lead to occlusion of diseased vessels and ischemia. Recanalization of a previously occluded vessel is often accompanied by reperfusion injury associated with damage to the affected tissue which triggers platelet recruitment to the site of injury. However, downstream signaling events may diverge from those leading to thrombus formation. Here, activation of FXII may also trigger formation of pro-inflammatory bradykinin which, in synergy with T-cells, contributes to a profound thrombo-inflammatory response, such as found in ischemic stroke. The mechanisms of T-cell recruitment and downstream effector molecules in this process are still elusive

significant numbers, develop ischemic infarcts, despite successful recanalization.

Detrimental Role of Platelets in Reperfusion Injury of the Brain

Platelet Tethering: GPIbα/VWF

To be able to adhere to the arterial wall under high shear flow conditions (> ~500 s^{-1}), platelets must be slowed down. This process, named tethering, depends on GPIb-V-IX, a structurally unique receptor complex exclusively expressed in platelets and megakaryocytes (Berndt et al. 2001) (Fig. 3). Platelet GPIbα binds to the A1 domain of immobilized von Willebrand factor (VWF) exposed on the surface of the vessel wall (Savage et al. 1996). VWF can be released from Weibel-Palade bodies of endothelial cells or α-granules of platelets (Kanaji et al. 2012). Although sufficient to support platelet binding, this adhesive interaction is characterized by a rapid dissociation rate. Firm platelet adhesion depends on cellular activation which is induced by other receptors such as the collagen receptor GPVI which is enabled to bind its ligand once the platelet is decelerated and in contact with the injury site (Nieswandt and Watson 2003) (Fig. 3). The VWF-binding site on GPIbα can be blocked in mice by Fab fragments of the antibody p0p/B which abrogates platelet tethering and adhesion under high shear flow conditions (Massberg et al. 2003). Treatment of mice with anti-GPIbα Fab prevented infarct development in ischemic stroke when given preventively before or therapeutically shortly after tMCAO during reperfusion (Kleinschnitz et al. 2007). Perfusion- and diffusion-weighted imaging employing ultrahigh-field magnetic resonance imaging (MRI) revealed that anti-GPIbα treatment secured permanent reperfusion after removal of the vessel-occluding filament, while in untreated mice blood flow steadily decreased leading to cerebral infarctions (Pham et al. 2011). Although tail bleeding times were strongly elevated in anti-GPIbα Fab-treated mice, no increase in ICH was detected histologically and on MRI scans, which represents the main obstacle for anti-thrombotic therapy during the acute stroke phase in clinical practice (Zinkstok et al. 2014). Moreover, the therapeutic effects were durable for at least 1-week follow-up after tMCAO (Kleinschnitz et al. 2007). Recently, these findings could be confirmed in aged and comorbid mice (Kraft et al. 2015). Atherosclerotic $Ldlr^{-/-}$, streptozotocin-treated diabetic, as well as hypertensive mice were likewise protected against reperfusion injury upon treatment with anti-GPIbα Fab after tMCAO. This is important because it has often been criticized that experimental findings in healthy laboratory animals do not reflect the real world situation in which stroke patients display multiple vascular risk factors that could modify or diminish treatment responses.

Phospholipase D (PLD) isoforms become activated downstream of major platelet signaling pathways including GPIbα, and PLD1 has been shown to be required for the formation of stable thrombi. Platelets from *Pld1*$^{-/-}$ mice displayed impaired $\alpha_{IIb}\beta_3$ integrin activation in response to major agonists and defective GPIbα-dependent aggregate formation under high shear flow conditions (Elvers et al. 2010). *Pld1*$^{-/-}$ mice were protected from ischemic stroke in the tMCAO model, whereas the animals showed normal tail bleeding times and, importantly, no ICH after stroke induction (Elvers et al. 2010). Pharmacological inhibition of PLD was similarly effective, opening a new and safe antithrombotic strategy in reperfusion injury of the brain downstream of GPIbα (Stegner et al. 2013b).

The critical role of GPIbα/VWF interactions in stroke development after tMCAO could further be substantiated in *Vwf*$^{-/-}$ mice. Infarct volumes were reduced by 60 % in these mice compared to wild-type control (Kleinschnitz et al. 2009), and reconstitution of plasma VWF by hydrodynamic gene transfer restored the susceptibility of mutant mice to cerebral ischemia (De Meyer et al. 2010). Further experiments using bone-marrow chimeric mice revealed that not only endothelial-derived VWF but also VWF stored in α-granules of platelets contributes to infarct development after tMCAO (Verhenne et al. 2015). In support of a major role of VWF/GPIbα interactions in cerebral ischemia, increased serum VWF concentrations have been found in the acute phase in patients (Kraft et al. 2014), and, moreover, VWF levels could be established as an independent stroke risk factor (Bongers et al. 2006).

Ultra-large VWF released from endothelial Weibel-Palade bodies into the circulation is rapidly cleaved by the enzyme "a disintegrin-like and metalloproteinase with thrombospondin repeats-13" (ADAMTS13) to reduce its high thrombotic activity (Sadler 2008). Thereby surplus VWF remote from a vascular lesion is cleared to avoid uncontrolled clotting and to limit thrombus growth. After 30 min of tMCAO, mice usually develop small infarcts restricted to the basal ganglia in contrast to an occlusion time of 1 h which leads to a complete MCA infarct within 24 h (Braeuninger et al. 2012). Thus, 30 min of tMCAO is a stroke model suitable for testing the role of factors expected to accelerate infarct development. In further support of an important pathophysiological role of VWF in stroke development, *Adamts13*$^{-/-}$ mice showed a dramatic increase in infarct volumes when compared to wild-type mice after 30-min tMCAO (Zhao et al. 2009; Fujioka et al. 2010). Vice versa, infusion of recombinant human ADAMTS13 into wild-type mice immediately before reperfusion reduced infarct volumes and improved neurological outcome in the 1-h tMCAO model (Zhao et al. 2009). Thus, blocking of GPIbα/VWF interactions or enhancing VWF clearance by ADAMTS13 consistently mitigated reperfusion injury after tMCAO and, remarkably, was not associated with bleeding complications despite increased tail bleeding times in these mice.

Momi and colleagues took this approach one step further toward a clinical application (Momi et al. 2013). They induced a complete MCA occlusion in guinea pigs by intravascular photothrombosis and treated the animals immediately with nanobodies directed against the A1 domain of VWF named ALX-0081. Surprisingly, ALX-0081, when given within 15 min after tMCAO in guinea pigs, facilitated thrombus dissolution and rapid, almost complete reperfusion similar to the fibrinolytic agent rt-PA which served as an active control substance and is commonly used in acute stroke patients. Independent from its thrombolytic effect at the site of vessel occlusion, ALX-0081 treatment in addition improved microvascular patency in the ischemic hemisphere and neurological outcome (Momi et al. 2013). Accordingly, disruption of platelet cross-linking by GPIbα/VWF inhibitors disaggregated the external layer of occlusive thrombi and restored vessel patency even at a stage when thrombi were resistant to fibrinolysis or traditional antithrombotic drugs (Le Behot et al. 2014). Collectively these studies suggest that GPIbα/VWF inhibitors may be suitable to accelerate recanalization during thrombolysis or mechanical thrombectomy and further protect the microvasculature during reperfusion as shown by ultrahigh-field MRI (Pham et al. 2011).

Platelet Adhesion/Activation: GPVI

GPIbα/VWF binding facilitates tethering of platelets to the vessel wall, but does not allow firm adhesion which requires cellular activation. At sites of endothelial damage, this is mainly mediated by GPVI, the principal activating platelet collagen receptor (Nieswandt and Watson 2003; Stegner et al. 2014) (Fig. 3). Upon activation GPVI non-covalently associates with the Fc receptor (FcR)γ-chain, and this complex signal through tyrosine phosphorylation cascades downstream of the FcRγ-chain-associated immunoreceptor tyrosine-based activation motif (ITAM) (Berlanga et al. 2002). Platelets in which GPVI has been depleted by antibody treatment in vivo do not respond to collagen (Nieswandt et al. 2001; Nieswandt and Watson 2003). To elucidate a putative role of platelet-collagen interactions during reperfusion injury of the brain, GPVI was either depleted in platelets by the anti-GPVI antibody JAQ1 (Kleinschnitz et al. 2007) or binding of platelets to the vessel wall was blocked by Revacept, a recombinant soluble dimeric GPVI-Fc, which occupies subendothelial collagen-binding sites for the receptor (Goebel et al. 2013). Both treatments led to a significant reduction of microvascular thrombus formation and reduced infarct volumes after tMCAO (Kleinschnitz et al. 2007; Goebel et al. 2013). These findings indicate that firm platelet adhesion is an

important step in the pathophysiology of reperfusion injury in the brain. In further support of this notion, elevated plasma levels of soluble GPVI were detected in patients with acute thrombotic stroke, but not in patients with transient ischemic attacks in which vessel-occluding thrombi rapidly and spontaneously dissolve (Wurster et al. 2013). Blocking of GPVI function can also be achieved by targeting tyrosine kinases and adaptor proteins downstream of GPVI (Stegner et al. 2014). The spleen tyrosine kinase (Syk) is such an essential signaling mediator downstream of the ITAM receptor GPVI (Poole et al. 1997). Accordingly, *Syk*$^{-/-}$ mice were protected from ischemic stroke and pharmacological blockade of Syk likewise ameliorated infarct development and clinical outcome (van Eeuwijk et al. 2016).

Platelet Aggregation and Formation of Occlusive Thrombi

Following initial adhesion of platelets to the extracellular collagen matrix, extension of the thrombus requires amplification of the initial platelet response, further recruitment of circulating platelets, and platelet aggregation which is mediated by GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) (Shattil and Ginsberg 1997) (Fig. 3) and other secretory platelet components (Nurden 2011). Earlier studies already described that partial pharmacological blockade of $\alpha_{IIb}\beta_3$ receptors in the tMCAO model reduced platelet and fibrin accumulation in the microvasculature as well as infarct volumes after tMCAO (Choudhri et al. 1998; Abumiya et al. 2000; Ishikawa et al. 2004). It became apparent, however, that the therapeutic window of $\alpha_{IIb}\beta_3$ inhibition was very narrow since near complete inhibition of platelet aggregation regularly led to large ICH. We reassessed efficacy and safety of anti-$\alpha_{IIb}\beta_3$ treatment in ischemic stroke by using $(Fab)_2$ fragments of the mouse $\alpha_{IIb}\beta_3$-blocking mAb, JON/A (Bergmeier et al. 2002) in young (Kleinschnitz et al. 2007) and aged, comorbid mice (Kraft et al. 2015). JON/A-$F(ab)_2$ treatment had no significant effect on peripheral platelet counts, but completely inhibited ex vivo platelet aggregation in response to different stimuli resulting in dramatically prolonged tail bleeding times (Bergmeier et al. 2002). In confirmation of previous studies, most mice with a virtually complete receptor blockade died mainly due to ICH, but, in contrast, the few surviving animals exhibited comparable infarct volumes as controls (Kleinschnitz et al. 2007). Reduction of $\alpha_{IIb}\beta_3$ blockade to 80 % or 70 % decreased bleeding-related mortality, but infarct volumes and neurological outcomes were not different from vehicle-treated mice. Similar results have been obtained in a clinical stroke trial that was prematurely stopped due to a dramatic increase in ICH in the anti-$\alpha_{IIb}\beta_3$-treated group (Adams et al. 2008). In transgenic mice lacking the GPIIb (and thus GPIIb/IIIa), cerebral infarct size was reduced at 24 h after tMCAO (Massberg et al. 2005). The reasons for these discrepant results are currently unclear, but it is conceivable that pharmacological blockade of $\alpha_{IIb}\beta_3$ could have different effects on reperfusion injury in the brain than $\alpha_{IIb}\beta_3$ deficiency. Further studies are necessary to clarify whether $\alpha_{IIb}\beta_3$-mediated platelet aggregation as the final step of platelet activation is involved in reperfusion injury of the brain or dispensable as hypothesized recently by us (Stoll et al. 2010; Nieswandt et al. 2011) (see "Platelet-Immune Interactions in Reperfusion Injury of the Brain").

Thrombus stability not only depends on platelet aggregation via $\alpha_{IIb}\beta_3$ but also on secretory platelet products. Platelets contain α- and dense granules which are released following adhesion to collagen or other matrix components as well as in response to agonists such as ADP and thrombin (Nurden 2011). Alpha-granules contain more than 300 proteins which are required for the propagation and stabilization of platelet-rich thrombi, while dense granules contain small, nonprotein molecules such as calcium, serotonin, ADP, and ATP. *Nbeal2*$^{-/-}$ mice, which lack α-granules and thereby reproduce the gray platelet syndrome, exhibit reduced adhesion on collagen and impaired thrombus growth at high shear rates and could not form occlusive thrombi in vascular injury models (Deppermann et al. 2013). Munc13-4 is a limiting factor for platelet granules release (Ren et al. 2010). Mice carrying an inactivating point mutation are not able to release dense granules, and α-granule release is diminished (Ren et al. 2010). In support of a critical role of granule release in reperfusion injury in the brain, both *Nbeal2*$^{-/-}$ and Munc13-4 mice were protected in the tMCAO model (Deppermann et al. 2013; Stegner et al. 2013a). An important component of the dense granule releasate is inorganic polyphosphate (PolyP), which acts as a potent activator of coagulation factor XII (FXII), the starting point of the intrinsic pathway of coagulation as well as inflammatory pathways through the formation of bradykinin (Muller et al. 2009). FXII deficiency or blockade was shown to markedly reduce cerebral infarct growth after tMCAO (Kleinschnitz et al. 2006; Hagedorn et al. 2010), but it remained unclear whether this protective effect was primarily based on reduced pro-coagulatory or pro-inflammatory activity in the acutely ischemic brain, or both (Fig. 3).

Platelets Are Gatekeepers of Hemostasis in the Ischemic Brain

Hemorrhagic transformation (HT) is a frequent complication of acute ischemic stroke, in particular after thrombolysis (Jickling et al. 2014) (Fig. 4a). In contrast to other organ systems, the brain is escluded from the circulation by the blood-brain barrier (BBB) which controls and limits the access of fluids and cells. As an early consequence of

cerebral ischemia, the BBB breaks down (Engelhardt et al. 2014; Knowland et al. 2014), mainly due to activation of matrix metalloproteinases which degrade the basal lamina around brain vessels (Hamann et al. 1996). The rate of HT is higher after transient MCAO with reperfusion than after permanent MCAO and further increases when reperfusion is delayed (Lu et al. 2009). Thus, there is a dilemma when blocking platelet functions during reperfusion in the brain: prevention of reperfusion injury may increase the risk of HT or overt ICH? As described above blockade of $\alpha_{IIb}\beta_3$ is associated with an extremely high risk of ICH in experimental (Kleinschnitz et al. 2007; Kraft et al. 2015) as well as clinical stroke (Adams et al. 2008; Kellert et al. 2013) and, even after ASA treatment in conjunction with thrombolysis, additional protective effects were offset by an unacceptable increase in ICH (Zinkstok et al. 2014). Accordingly, baseline antiplatelet use is associated with an increased risk of post-rt-PA symptomatic ICH in acute stroke (Cucchiara et al. 2009). This also applies to the intact, non-ischemic brain: improved efficacy of platelet inhibition in cardio- or cerebrovascular disorders, e.g., arterial stenoses and stenting, is often neutralized by a higher incidence of ICH (Gachet 2015) (Fig. 4b). Recently, it was reported in patients that a subset of procoagulant platelets, so-called COAT platelets, were increased in non-lacunar strokes, and lower levels were associated with acute hemorrhagic complications (Prodan et al. 2015) indicating a putative role in stroke hemostasis. It is therefore important to understand how platelets act to prevent ICH especially in the stroke-injured brain.

The most simple question one could imagine is how many platelets are needed for hemostasis. We induced thrombocytopenia of variable degree in mice by injection of polyclonal anti-GPIbα antibodies which deplete circulating platelets in mice without involving immune effector mechanisms (Nieswandt et al. 2000). Mice with significantly reduced platelet counts down to a range of 99–25 platelets/nL were still able to arrest bleeding in the tail bleeding test, and only after reduction to <2.5 % of control, bleeding no longer stopped spontaneously (Morowski et al. 2013). When thrombocytopenic mice were challenged by tMCAO, full-blown MCA infarcts developed unless platelet counts were reduced <10 % of normal (Morowski et al. 2013). Importantly, no HT or ICH was observed in these stroke-protected mice despite severe thrombocytopenia. These findings indicate that significant thrombocytopenia does not protect from reperfusion injury in the brain and that a very low count of intact platelets is sufficient to maintain hemostasis similar to clinical observations in idiopathic thrombocytopenic purpura (Neunert et al. 2013). Goerge and coworkers further showed that the propensity of thrombocytopenic mice to bleed depends on the local tissue environment (Goerge et al. 2008). Thrombocytopenic mice did not bleed spontaneously, but major hemorrhages occurred in the skin and lung when an additional local inflammatory stimulus was set. As outlined in detail below, ischemic stroke exhibits a significant extrinsic inflammatory component (Stoll et al. 1998). In support of the concept that inflammation predisposes to HT, thrombocytopenic mice (<2.5 % of normal) undergoing tMCAO exhibited multiple hemorrhagic foci (Goerge et al. 2008). In the skin, the presence of platelets prevented bleeding during inflammation, and this protective effect was unexpectedly also seen in mice lacking functional GPIbα, VWF, or GPVI indicating that tethering and firm adhesion of platelets via classical adhesion receptors were not required for hemostasis. This is in accordance with studies in the tMCAO model, in which mice lacking GPIb, VWF, or GPVI were stroke protected, but did not develop ICH (Kleinschnitz et al. 2007, 2009). Thus, it appears that the mechanisms by which platelets contribute to the pathogenesis of ischemic brain injury are different from those required to maintain hemostasis following ischemia/reperfusion which raises the intriguing possibility that strong platelet inhibition can be achieved without dramatically increasing the risk of (spontaneous) intracranial

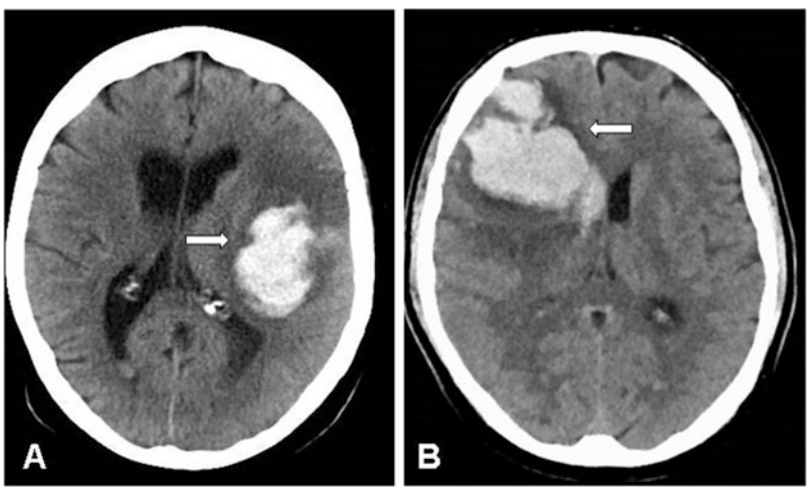

Fig. 4 The brain is vulnerable to intracranial hemorrhage (ICH). (**a**) CT scan of an ICH in the left hemisphere (*white* area marked by *arrow*) occurring spontaneously after thrombolysis using rt-PA. (**b**) Shows a frontally located ICH (*white* area marked by *arrow*) which occurred after arterial stenting under double platelet inhibition by ASA and ticagrelor

bleeding. Studies in the skin and lung moreover showed that platelets lacking G protein-coupled receptor signaling, thereby not responding to thrombin, ADP, and thromboxane A_2, were still protected against inflammation-induced bleedings, while blocking of the ITAM signaling pathway led to massive hemorrhages (Boulaftali et al. 2013). It is unclear whether this also applies to ischemic stroke. Interestingly, lack of Munc13-4 resulted in severely defective platelet aggregate formation (Savage et al. 2013), but mice did not develop increased ICH after tMCAO, in sharp contrast to mice with massive ICH in a parallel group in which $\alpha_{IIb}\beta_3$ was blocked (Stegner et al. 2013a). To improve stroke outcome and safety during recanalization procedures, it is mandatory to understand which platelet functions secure hemostasis during acute brain ischemia, a so far widely neglected field in experimental stroke research. At subacute and late stroke stages beyond 24 h, additional factors such as proteases, vascular remodeling (Jickling et al. 2014), and macrophage responses (Gliem et al. 2012) contribute to HT or prevent it, but at this stage the use of antiplatelet drugs is less critical and of proven value for secondary stroke prevention (Sandercock et al. 2014).

Platelet-Immune Interactions in Reperfusion Injury of the Brain

Brain ischemia has traditionally been regarded as a pure thrombotic disorder, but already more than 20 years ago, experimental and human studies revealed that immune cells infiltrate the brain after cerebral ischemia, and cytokines which orchestrate immune responses are upregulated within the ischemic brain parenchyma (Stoll et al. 1998; Lambertsen et al. 2012). The complex immune cell-brain interactions during the subacute and chronic phases of ischemic stroke have been reviewed elsewhere (Iadecola and Anrather 2011; Chamorro et al. 2012); here we focus on the role of T-cells and platelets during reperfusion early after cerebral ischemia (Fig. 3). In a seminal study, Yilmaz and colleagues showed that immune-deficient *Rag1*$^{-/-}$ mice which lack B- and T-cells are protected against cerebral ischemia in the tMCAO model (Yilmaz et al. 2006). These surprising data were soon confirmed thereafter (Kleinschnitz et al. 2010). Both studies revealed that adoptive transfer of T-cells into *Rag1*$^{-/-}$ mice restored susceptibility to ischemic brain damage, while B-cell transfer had no effect. A detailed immunological analysis showed that the detrimental T-cell effects in reperfusion injury of the brain largely were antigen independent and conferred by all major subclasses of T-cells, e.g., $CD4^+$, $CD8^+$, natural killer T-cells, etc. Surprisingly, also regulatory T-cells were deleterious (Kleinschnitz et al. 2013), and boosting of regulatory T-cells by CD28 agonist further increased ischemic brain damage (Schuhmann et al. 2015). FTY720 is an immunomodulator clinically approved for treatment of multiple sclerosis patients which rapidly reduces peripheral lymphocyte counts by blocking the egress of lymphocytes from lymphoid organs. FTY720, when given immediately before reperfusion in the tMCAO model, improved outcome (Kraft et al. 2013), a finding that could also be reproduced in a pilot trial of acute stroke patients who were treated by thrombolysis and in addition received FTY720 (Zhu et al. 2015). Thus, although it is too early for firm conclusions, it appears that T-cell responses are also critically involved in acute human stroke. The profound detrimental effect of T-cells is not unique for brain ischemia but rather represents a general pathophysiological phenomenon during organ reperfusion (Eltzschig and Eckle 2011). Importantly, thrombus formation itself was not altered in stroke-protected *Rag1*$^{-/-}$ mice as assessed in two standardized in vitro and in vivo thrombus formation models. Platelets from *Rag1*$^{-/-}$ mice normally adhered to collagen fibers and formed aggregates on collagen-coated surfaces in a whole-blood perfusion system under high shear conditions. In addition, vessel occlusion times were identical compared to immune-competent mice after $FeCl_3$-induced injury on mesenteric arterioles (Kleinschnitz et al. 2013). Taken together, these findings showed that brain damage after ischemia cannot merely be explained by a thrombotic event in the microcirculation. Taking into account the unequivocal contribution of T-cells to reperfusion injury, we coined the term "thrombo-inflammation" (Stoll et al. 2010; Nieswandt et al. 2011) (Fig. 3), a concept supported by the fact that detrimental T-cell effects are platelet dependent (Kleinschnitz et al. 2013). We could show that *Rag1*$^{-/-}$ mice were still protected from ischemic brain injury when platelets had been removed before adoptive transfer of detrimental T-cells (Kleinschnitz et al. 2013). Platelets can influence lymphocyte function and vice versa (Li 2008). In the liver $CD4^+$ T-cells have been shown to interact with platelets in postischemic sinusoids during reperfusion via platelet CD62P (P-selectin) (Khandoga et al. 2006). The molecular mechanisms underlying the concerted detrimental actions of T-cells and platelets in the brain are still elusive, but the encouraging results obtained by both experimental studies and a recent clinical pilot trial warrant further investigations to disclose the mystery of reperfusion injury and the paradoxically harmful restoration of blood flow in the brain.

Take-Home Messages

- Successful restoration of cerebral blood flow by thrombolysis and/or mechanical thrombectomy is the mainstay of acute stroke treatment, but recanalization alone does not guarantee a favorable outcome.

(continued)

- Reperfusion injury denotes the acute, paradoxically harmful aspect of blood flow return in the ischemic brain which involves platelet activation and immune cell recruitment.
- In experimental animals, blocking of platelet GPIbα or GPVI, as well as reducing circulating VWF, dramatically improved stroke outcome by protecting the microvasculature during reperfusion and further accelerated recanalization during thrombolysis without bleeding complications.
- Contrastingly, interfering with platelet aggregation by blocking $\alpha_{IIb}\beta_3$ led to devastating intracranial hemorrhages in acute experimental and human stroke.
- Before clinical application it is essential to further dissect pathological platelet functions and activation pathways involved in reperfusion injury from those indispensable as gatekeepers of hemostasis in the stroke-injured brain.
- The pathophysiology of reperfusion injury in acute stroke is even more complex and involves concerted detrimental actions of platelets and T-cells, referred to as "thrombo-inflammation," which awaits further elucidation.

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Platelets in Deep Venous Thrombosis and Pulmonary Embolism

Ingrid Pabinger, Julia Riedl, and Simon Panzer

Abstract

Platelets have important roles in the development of venous thromboembolism (VTE). In experimental studies, including animal models of venous thrombosis, platelets were found to augment thrombus formation in the venous system, and the importance of the interaction between platelets and the plasmatic coagulation system for the development of VTE was shown. Furthermore, studies on patients with acute VTE and on patients at high risk for VTE have revealed that markers of platelet activation are elevated in these settings, and platelet markers, such as soluble P-selectin, might serve as biomarkers of acute VTE or to indicate patients with a high risk of VTE. Most convincingly, antiplatelet therapy with aspirin was found to reduce risk of VTE after orthopedic surgery and risk of recurrent VTE after a first event and might be an additional therapeutic option. The therapeutic effect of aspirin in the prevention of VTE proves the concept that platelets are important players in thrombosis of the venous system.

Introduction

Platelets and platelet activation are best known for their key role in the pathophysiology of arterial thrombosis, and antiplatelet therapy is a main concept in the treatment of atherothrombotic diseases. The situation is less clear in deep vein thrombosis (DVT) and pulmonary embolism (PE), together referred to as venous thromboembolism (VTE). Platelet involvement in VTE has long been viewed to have a minor role, as venous thrombi consist of a fewer amount of platelets in comparison to arterial thrombi. However, today, growing evidence suggests an important role of platelets also in VTE. Experimental studies show that platelets are essential in initiation and augmentation of venous thrombosis. Platelet indices, such as mean platelet volume, were found to be associated with the risk of VTE, and most convincingly, randomized clinical trials for the evaluation of antiplatelet agents in VTE prophylaxis show an effective reduction in the risk of VTE after orthopedic surgery and a reduced risk of VTE recurrence after a first episode if patients have been treated with aspirin. This chapter highlights the most important findings on the role of platelets in VTE.

Deep Venous Thrombosis and Pulmonary Embolism

VTE is the third most frequent cause of vascular death after myocardial infarction and stroke worldwide. The mean incidence of VTE is approximately 1–2 per 1000 per year and increases exponentially with age (Naess et al. 2007; ISTH Steering Committee for World Thrombosis Day 2014). The etiology of VTE is multifactorial, and this was first described in the nineteenth century by Rudolf Virchow, who proposed that three factors are crucial for the development of VTE

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(known as "Virchow's triad"): alterations in blood flow ("stasis"), alterations of the blood components, and damages to the vessel wall. Today, multiple risk factors for VTE have been identified (Reitsma et al. 2012) that are related to one of the three categories of "Virchow's triad": Stasis of the venous blood flow after immobilization, for example, is a well-known risk factor of VTE. Thrombophilic changes of blood components, such as genetic mutations of coagulation factors (factor V Leiden mutation, prothrombin mutations, etc.), were identified to be associated with risk of VTE. Finally, in experimental studies endothelial activation was found to be crucial for the formation of a clot (Mackman 2012). In conclusion, VTE is a multifactorial disease, influenced by multiple inherited and acquired risk factors. This chapter focuses on the contribution of platelets, the cellular part of the coagulation system, to the development of VTE.

Platelets in the Pathophysiology of Deep Venous Thrombosis and Pulmonary Embolism

From a classical point of view, platelets are key mediators in the pathophysiology of thrombosis in the arterial system, whereby they attach to injured vessel walls after rupture of an atherosclerotic plaque under high shear stress and induce formation of a platelet-rich "white" clot (Mackman 2008). In contrast, venous thrombi are proposed to form over an intact vessel wall in a low shear system through multifactorial processes and are rich in fibrin and trapped red blood cells ("red clots") (Mackman 2008). However, platelet hyperaggregability and shortened platelet survival in patients with deep vein thrombosis were described already decades ago (Wu et al. 1976). In addition, the presence of platelets in venous thrombi (Sevitt 1970) suggests their involvement in thrombus formation. Activated platelets interact with the plasmatic coagulation system, exposing the main fibrinogen receptor $\alpha_{IIb}\beta_3$, which also serves as a receptor for von Willebrand factor, and other important plasma coagulation proteins. Further, the platelet glycoprotein (GP) Ibα, of the platelet GPIb-IX-V complex, binds von Willebrand factor (VWF) or thrombospondin (TSP) and is co-associated with the platelet-specific collagen receptor, GPVI (Arthur et al. 2005). The GPIb-IX-V/GPVI adhesion-signaling complex initiates platelet activation and aggregation and thus thrombus formation in response to vascular injury and collagen exposure. GPIbα also regulates coagulation through a specific interaction with thrombin and other coagulation factors, reviewed in Gardiner and Andrews (Gardiner and Andrews 2014). Activated platelets expose negatively charged phospholipids and provide a procoagulant surface, thereby promoting the generation of thrombin (Heemskerk et al. 2002). In a rat model, thrombus formation was reduced in thrombocytopenic animals as compared to animals with a normal platelet count. In this model, thrombus formation was induced by ligation of the inferior vena cava and infusion of low dosage of tissue thromboplastin. Platelet reduction did not inhibit thrombus formation with high tissue thromboplastin concentrations; however, an 87 % reduction of thrombus formation with low concentration of tissue thromboplastin was observed, pointing to an important role of platelets at least at mild thrombogenic stimuli (Herbert et al. 1992). Another study on VTE in the rat showed that the antiplatelet agents aspirin and clopidogrel reduce thrombus formation after adriamycin-induced VTE (Bernat and Herbert 1994). In a more recent study, the interaction between platelet GPIbα and von Willebrand factor (vWF) was found to be a crucial step in a mouse model of VTE. In this model, DVT was induced by flow restriction in the inferior vena cava only (Brill et al. 2011). Furthermore, platelets propagate the formation of venous thrombi by recruiting and interacting with leukocytes and by inducing the release of neutrophil extracellular traps (NETs) (von Brühl et al. 2012). In the latter study, a mouse model of DVT by flow restriction in the inferior vena cava was combined with intravital imaging techniques, allowing in vivo tracking of platelets during thrombus formation. This study showed that platelets are recruited to the developing thrombus very early after flow restriction and support the accumulation of leukocytes. Further, GPIbα was essential for the effects of platelets in propagating thrombosis, pointing to an important role of platelet GPIbα in VTE.

Platelet Biomarkers in Deep Venous Thrombosis and Pulmonary Embolism

Multiple studies focused on the evaluation of platelet count, platelet volume, and soluble markers of platelet activation for risk prediction of first or recurrent VTE and on the value of these parameters for diagnosis and prognosis of VTE.

Biomarkers of Increased Platelet Activation

Currently, the most extensively studied biomarker of platelet activation is soluble P-selectin (sP-selectin). P-selectin is an adhesion molecule that is located in the alpha-granule of resting platelets and that becomes translocated to the platelet surface upon platelet activation and degranulation. Once on the surface, it is cleaved and a soluble part, sP-selectin, is released into the circulation. sP-selectin in plasma can also derive from the Weibel-Palade bodies of endothelial cells upon their activation, but the bulk amount of sP-selectin is platelet derived (Fijnheer et al. 1997; Jilma-Stohlawetz et al. 2014). Levels of sP-selectin are elevated in patients with acute VTE (Smith et al. 1999; Rectenwald et al. 2005; Gremmel

et al. 2011) and in patients with recurrent VTE (Ay et al. 2007). A meta-analysis including 11 studies, comprising 586 VTE patients and 1843 controls, reconfirmed that sP-selectin is significantly elevated in acute VTE and concluded that sP-selectin might be a biomarker for the diagnosis of VTE (Antonopoulos et al. 2014). High sP-selectin levels also predict risk of recurrent VTE after a first unprovoked event (Kyrle et al. 2007) and risk of VTE in cancer patients (Ay et al. 2008). However, despite numerous data pointing to an important role of sP-selectin for a diagnosis and risk prediction of VTE, there are so far no rapid and standardized methods for its measurement in clinical routine, currently limiting its clinical applicability as a marker (Blann 2014).

Another biomarker of platelet activation is the soluble form of CD154 or CD40 ligand (CD40L). CD40L is located in the platelet alpha-granules and becomes translocated to the platelet surface upon their activation. Once on the surface, it is cleaved and a soluble form of CD40L (sCD40L) is released into the circulation. sCD40L can be measured in plasma as a biomarker of platelet activation. The CD40-CD40L axis has important functions in the adaptive immune system, such as activation of antigen-presenting cells, and the discovery of CD40L on platelets points to a role of platelets in adaptive immunity (Elzey et al. 2011). sCD40L was found to be elevated in patients with acute coronary syndrome, and in these patients, it is associated with worse clinical outcome (Ferroni et al. 2012).

Two studies investigated levels of sCD40L in patients with VTE. Levels of sCD40L were higher in patients with acute PE compared to healthy controls (Kaya et al. 2011), whereas sCD40L levels were not different in patients with a history of spontaneous VTE and controls (Migliacci et al. 2007).

In a matched nested case-control study, embedded within an observational study on cancer patients, levels of sCD40L, measured before the beginning of chemotherapy, were not found to be associated with risk of cancer-associated VTE (Riedl et al. 2015).

In conclusion, current data suggest that there is no role for measuring sCD40L to predict risk of VTE; however, it might be a biomarker of acute VTE.

Platelet factor (PF)-4 is another important biomarker of platelet activation, which is stored in the platelet alpha-granules, from where it is released upon platelet activation. It was found to have procoagulatory activity in vitro; however, its biological function in vivo is not yet fully understood (Kowalska et al. 2010). In a clinical study, levels of PF-4 were found to be increased in patients with acute VTE (Blanke et al. 1987). Another study found high amounts of PF-4 to be associated with an increased risk of VTE in patients with pancreatic cancer (Poruk et al. 2010). However, a study by our group found no association between levels of PF-4 and risk of VTE in patients with different types of cancer, (Riedl et al. 2015) suggesting a minor role of PF-4 for risk prediction of VTE. It has to be noted, however, that release of PF-4 is prone to artificial platelet activation during sample processing, which might explain diverging results of studies and which also currently further limits the clinical applicability of PF-4 as a biomarker of VTE.

Platelet Count

In the general population, platelet count is not a risk factor for VTE, which was shown in large observational studies on community-dwelling individuals that found no relationship between platelet count and risk of first VTE event (Tsai et al. 2002; van der Bom et al. 2009).

High Platelet Counts and Risk of VTE

Cancer patients are at high risk for developing VTE, with an estimated risk of up to 20 % per year, depending on cancer site, stage, and several other factors. Platelets seem to have a very special role in the development of VTE in cancer patients. Cancer cells have the ability to activate and aggregate platelets directly, and thus they are anticipated to be involved in VTE formation and also in the progression of the malignant disease [reviewed in Riedl et al. (2014), illustrated in Fig. 1]. In cancer patients, an elevated platelet count, measured prior to the initiation of chemotherapy, was found to be associated with risk of VTE (Khorana et al. 2005; Simanek et al. 2010). In the Vienna Cancer and Thrombosis Study (CATS), the risk of patients who had a platelet count above the 95th percentile of the whole cohort (>443 G/l) had a fivefold higher risk to develop VTE compared to patients with a platelet count below that cutoff (Simanek et al. 2010). A platelet count of 350 G/l or more has also been incorporated into a risk prediction score to identify cancer patients undergoing chemotherapy in the outpatient setting who are at a high thrombotic risk (Khorana et al. 2008). Interestingly, data from a population-based study from Norway revealed that a high platelet count, measured several years before the diagnosis of cancer, is associated with an elevated risk of VTE in subjects who later develop cancer (Jensvoll et al. 2014). In contrast, platelet count was not associated with risk of VTE in subjects who remained cancer-free, and it was concluded that platelet count might specifically play a role in the pathogenesis of cancer-associated VTE.

Very high platelet counts (>1000 G/l) are often seen in patients with essential thrombocythemia (ET), a myeloproliferative disorder (MPN) with increased platelet production and high risk of arterial and venous thrombosis. Interestingly, extreme thrombocytosis was not found to be a risk factor for VTE in these patients but rather for bleeding events, as very high platelet counts can cause acquired von Willebrand syndrome (vWS) (Reikvam and Tiu 2012; Kreher et al. 2014). This assumption that a relative shortage of von Willebrand factor ensues if platelet counts are high

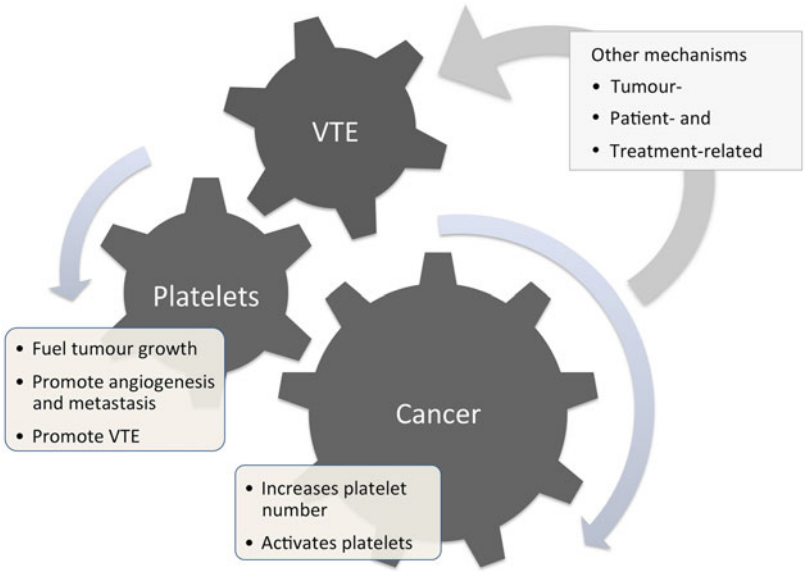

Fig. 1 Proposed mechanisms of how cancer and platelets influence each other and thereby contribute to the development of VTE: As VTE is a multifactorial disease, platelets are only one aspect of how hypercoagulability develops in cancer

and thus consuming all von Willebrand factor is further exemplified in another observation: In more than 40,000 patients, who received anticoagulatory drugs for acute VTE, a very low (<80 G/l) as well as a very high (>450 G/l) platelet count were associated with a high risk of bleeding in comparison to normal platelet counts (Di Micco et al. 2013).

Low Platelet Counts and Risk of VTE

In certain diseases, a low platelet count might also be associated with an increased risk of VTE. This is obviously the case in disseminated intravascular coagulation, where arterial but even more frequent venous thromboembolic events might occur (Wada et al. 2013). Also in a certain subgroup of cancer patients, those with high-grade glioma, a low platelet count was found to be a risk factor for VTE and was also an important parameter in development of a score to identify patients with a high risk of VTE. The score contains platelet count (<196 G/l) and soluble p-selectin (>51.9 ng/ml). The cumulative VTE probability after 12 months was 9.7 % for score 0 ($n = 76$, neither low platelet count nor elevated sP-selectin), 18.9 % for score 1 ($n = 59$) and 83.3 % for score 2 (low platelet count and elevated sP-selectin $n = 6$) (Thaler et al. 2014). Thus this score, which contains numbers of platelets and a soluble platelet activation marker, could be of help to identify high-grade glioma patients at low risk or high risk of VTE.

Another disease that is associated with a slightly increased risk of VTE despite a low platelet count is immune thrombocytopenia (ITP) (Rodeghiero 2015). During the studies with thrombopoietin-stimulating agents (TPAs), a slightly increased risk of venous thrombotic events was observed in the treatment group but also in the group of patients on standard care (Kuter et al. 2010; Rodeghiero et al. 2013; Rodeghiero and Ruggeri 2015). However, major risk factors for VTE in ITP seem to be higher age, splenectomy, and personal risk factors (Rodeghiero 2015). Higher reactivity of platelets is thought to be the underlying mechanisms for VTE in ITP patients (Psaila et al. 2011). The activation of platelets during treatment with TPAs was studied in two prospective studies (Psaila et al. 2012; Haselboeck et al. 2013). Activation of platelets was seen in non-treated patients, those on corticosteroids, and those on TPAs, and the activation was not more pronounced in eltrombopag-induced platelets (Haselboeck et al. 2013), indicating that platelet activation alone does not suffice for the risk for VTE in ITP patients.

Mean Platelet Volume

The mean platelet volume (MPV), a parameter which indicates platelet size, was proposed to reflect platelet activity, as it is associated with platelet aggregation, (Thompson et al. 1982) release of thromboxane B2 (Jakubowski et al. 1983), and increased expression of the platelet adhesion molecule and fibrinogen receptor $\alpha_{IIb}\beta_3$ (Giles et al. 1994). Clinical studies have shown that high MPV is a risk factor for a variety of thromboembolic diseases (Leader et al. 2012), such as myocardial infarction (Slavka et al. 2011) or stroke (Greisenegger et al. 2004; Bath et al. 2004), and for increased risk of overall vascular disease-associated mortality (Slavka et al. 2011). High MPV was also found to be associated with the risk of developing VTE in a large population-based study from Norway, especially with the risk of unprovoked VTE

(Brækkan et al. 2010). In contrast, studies on cancer patients revealed that a high MPV in those patients is associated with lower risk of cancer-associated VTE (Riedl et al. 2013; Ferroni et al. 2014). In conclusion, platelet size seems to be relevant for the development of VTE, but the influence of platelet size on VTE risk differs between patient subgroups (idiopathic VTE vs. cancer-associated VTE).

Pharmacological Inhibition of Platelet Function for Prevention and Treatment of Deep Venous Thrombosis and Pulmonary Embolism

Pharmacological interventions for treatment and prevention of VTE are mainly based on administration of anticoagulants including heparins, vitamin K antagonists, and novel, direct oral anticoagulants (DOACS). These substances are regarded first choice for the prevention and treatment of VTE. However, evidence has been presented that antiplatelet agents, specifically aspirin, can also be effective in primary prevention of VTE in surgery and for secondary prevention of VTE after a first event of venous thrombosis or pulmonary embolism.

Primary prevention of VTE is recommended in a variety of clinical situations and diseases with increased risk of VTE. The most important one is surgery, and among the surgical procedures, orthopedic and tumor surgery bear the highest risk of VTE. This chapter will only highlight the role of aspirin and not of anticoagulants for prevention of venous thromboembolic events. The effect of aspirin on a decrease of the risk of VTE is regarded as plausible due to the effect of platelets on thrombus formation and thrombus growth, which has been outlined in the introduction and discussed in the literature (Becattini et al. 2012).

There are only very limited and no recent studies in general surgery in which aspirin was systematically investigated. Data on the efficacy and safety of aspirin in non-orthopedic, non-traumatic surgery date back to the 1980s. These studies were reviewed by the Antiplatelet Trialists' Collaboration in 1994 (Cohen et al. 1994). Diagnosis of deep vein thrombosis at that time was made either by systematic radiolabeled fibrinogen uptake scan or by venography.

In the aspirin group, 178/1434 (19.4 %) patients and in the control group (open or placebo) 369/1459 (27.1 %) developed objectively confirmed deep vein thrombosis, which means an odds reduction of 37 % (8 % standard deviation) for aspirin. When pulmonary embolism was evaluated 16/3408 (0.5 %) on aspirin and 58/3419 (1.7) controls developed pulmonary embolism, which means a 71 % (with 14 % standard deviation) odds reduction, differences were statistically significant. The Antiplatelet Trialists' Collaboration group evaluated rate of bleeding together in trials that included general and orthopedic surgery. There was an increase of transfusion requirements and also other complications, such as hematoma or wound infections in the aspirin group.

One randomized double-blind study comparing aspirin with unfractionated heparin was conducted by Vinazzer et al. in patients with elective general surgery (Vinazzer et al. 1980). Five hundred mg of aspirin were compared to 5000 IU of unfractionated heparin twice daily, 1210 patients were included into that study. Deep vein thrombosis as the primary outcome was based on obligatory Doppler imaging. No statistically significant difference was found in the rates of deep vein thrombosis (3.9 versus 2.4 %) or pulmonary embolism (0.3 each), and the risk of bleeding was also similar (0.7 % each). Due to lack of data with more recent high-quality and large studies, aspirin cannot generally be recommended as thrombosis prophylaxis in general surgery and of course also not in tumor surgery.

There is one very large study (pulmonary embolism prevention = PEP trial) in patients with hip fracture surgery (13.356 patients) or elective hip replacement (5.088 patients) surgery. This is the largest study performed so far in orthopedic/traumatic surgery. Patients received either 160 mg aspirin or placebo for 35 days in routine care, and additional thrombosis prophylaxis was allowed, if deemed necessary by the investigator. Only symptomatic events were recorded and an independent blinded committee adjudicated the events. Patients with hip fracture surgery on aspirin had a significantly reduced rate of PE compared to those on placebo; the effect on DVT was also a significant reduction. Taken together, aspirin lead to a proportional reduction of PE and DVT of 36 %. Deaths due to bleeding were not increased in patients with aspirin; however, bleeding requiring transfusion was significantly ($p = 0.04$) increased. Remarkably, death from PE was reduced significantly in the aspirin group (18 in the aspirin group versus 43 in the placebo group), which corresponded to a 58 % rate reduction of fatal PE ($p = 0.02$). In other words, aspirin prevented four fatal PEs per 1000 patients. Due to perceived changes in the primary outcome and changes in the sample size during the study, this trial was criticized (Falck-Ytter et al. 2012). However, despite some limitations of the study design, aspirin is regarded as option for thrombosis prophylaxis in orthopedic surgery by a number of specialists and in guidelines (Falck-Ytter et al. 2012).

There are several clinical conditions with considerably increased risk of VTE also in nonsurgical patients. The highest risk can be found in cancer patients with a probability to develop VTE of up to 30 % after 6 months in certain subgroups (Ay et al. 2010). An easy and convenient prevention would be a big advantage for cancer patients at high risk of VTE. Aspirin would fulfill these criteria, but its potential in preventing VTE in this patient population has not been specifically investigated in patients with solid tumors, so far.

However, in observational studies, no effect on the frequency of VTE in patients, who were on aspirin for other reasons, was found (Lötsch et al. 2014).

Patients with multiple myeloma who are treated with thalidomide or lenalidomide in combination with chemotherapy and/or dexamethasone have a high risk of VTE, and therefore primary thromboprophylaxis is recommended. In patients with multiple myeloma, thromboprophylaxis with aspirin was proven to be as effective as low-molecular weight heparin (Larocca et al. 2012) and was studied and compared to LMWH and warfarin in patients with multiple myeloma on thalidomide and chemotherapy (Palumbo et al. 2011). Although within the first 3–6 months the VTE risk was lowest in patients on LMWH, no difference between the three groups was observed after 9 months. Thus aspirin is frequently used as thrombosis prophylaxis in patients with multiple myeloma during antineoplastic treatment with thalidomide or lenalidomide in combination with chemotherapy and/or dexamethasone.

Furthermore, patients with MPNs, such as ET or polycythemia vera (PV), have a high risk of vascular events and should receive primary thromboprophylaxis. Current guidelines recommend the use of aspirin in PV and in ET patients, which harbor the JAK2 mutation (Kreher et al. 2014). In PV, these recommendations are based on a randomized trial that showed a significantly reduced rate of cardiovascular events and also a reduction—although not statistically significant—in VTE events (Landolfi et al. 2004). In ET, conclusive data are lacking; however, a retrospective analysis showed that in the subgroup of patients with the JAK2 mutation, aspirin significantly reduced the risk of VTE (Alvarez-Larrán et al. 2010). Aspirin should not be given to patients with platelet counts > 1000 G/l or acquired vWS, as these patients have a high bleeding risk.

Patients with antiphospholipid antibodies have also an increased risk of VTE, and primary prevention would be an option in high-risk patients. One placebo-controlled trial, the Antiphospholipid Antibody Acetylsalicylic Acid (APLASA) study investigated the efficacy and safety of aspirin in 98 asymptomatic patients with persistently positive antiphospholipid antibodies comparing aspirin 81 mg daily vs. placebo for prevention of arterial or venous thrombosis (Erkan et al. 2007). Aspirin was not superior to placebo with regard to new onset of venous or arterial thrombosis (HR, 1.04; 95 % CI, 0.69–1.56), but bleeding was also not increased.

A subgroup analysis of the Women's Health Study of asymptomatic individuals with hereditary thrombophilia (factor V Leiden, prothrombin G20210A variation) failed to demonstrate an effect of aspirin on the rate of VTE (HR, 0.83; 95 % CI, 0.50–1.39) (Glynn et al. 2007).

Aspirin did not prevent flight-associated VTE compared to control; however, long-term data are not available as follow-up of the study participants ended when they left the airport (Cesarone et al. 2002).

Prevention of Recurrent Deep Venous Thrombosis and Pulmonary Embolism

Two recent, randomized placebo-controlled double-blind, multicenter trials evaluated the effect of aspirin for the prevention of recurrent VTE after completion of an initial course of anticoagulant treatment. Both studies had a similar design and compared aspirin, 100 mg once daily, to placebo in patients with unprovoked VTE who had completed initial anticoagulant therapy: The Warfarin and Aspirin (WARFASA) study assessed the benefit of aspirin, given over a 2-year period, in comparison to placebo and found a significantly reduced risk of VTE in the aspirin group (6.6 % vs. 11.2 %) with no increase in the risk of major bleeding (Becattini et al. 2012). The second trial, Aspirin to Prevent Recurrent Venous Thromboembolism (ASPIRE), had a longer study period of up to 4 years and found no significant reduction in the VTE rate in the aspirin group [4.8 % per year for aspirin vs. 6.5 % per year for placebo; hazard ratio (95 % confidence interval) with aspirin 0.74 (0.52–1.05), $p = 0.09$] (Brighton et al. 2012). However, the combined rate of major vascular events in this trial, including VTE, myocardial infarction, stroke, or cardiovascular death, was reduced significantly by 34 %, without increasing the risk of bleedings.

Moreover, a predefined meta-analysis of data from both trials was performed and separately published (Simes et al. 2014). Key data from the two trials as well as from the combined analysis are shown in Table 1. In the combined

Table 1 Summary and key data from two recent randomized, controlled trials comparing low-dose aspirin (100 mg daily) vs. placebo for the prevention of recurrent VTE after completion of an initial anticoagulant treatment episode for a first episode of unprovoked VTE, as well as data from the predefined combined analysis of the two studies

Study name	Total number of patients randomized	Median follow-up period (months)	Risk of VTE/year, aspirin vs. placebo [HR for aspirin (95 % Confidence interval)]	Risk of major bleeding/year, aspirin vs. placebo
WARFASA	403	23.9	6.6 % vs. 11.2 % [0.58 (0.36–0.93), $p = 0.02$]	0.3 % vs. 0.3 %
ASPIRE	822	37.2	4.8 % vs. 6.5 % [0.74 (0.52–1.05), n.s.]	1.1 % vs. 0.6 % (n.s.)
Combined analysis	1224	30.4	5.1 % vs. 7.5 % [0.68 (0.51–0.90), $p = 0.008$]	0.5 % vs. 0.4 % (n.s.)

n.s. not significant

analysis, the authors showed a significant risk reduction for VTE in aspirin users, and altogether it was concluded that aspirin can safely reduce the recurrence rate of VTE by approximately 30 % (Simes et al. 2014). Of interest, the absolute rate of fatal recurrent VTE in patients on aspirin (0.16 per 100 patient-years, 95 % confidence interval (CI) 0.03–0.40) was as low as in those on direct oral anticoagulants (DOACS) (0.18 per 100 patients-years, 95 % CI, 0.06–0.37) and vitamin K antagonists (0.14) per 100 patient-years (95 % CI, 0.04–0.32). In the placebo group the rate of fatal VTE was approximately twice as high (0.38 per 100 patient-years, 95 % CI 0.18–0.65) (Wu et al. 2015). Thus, with regard to occurrence of a fatal VTE after a first VTE, aspirin seems not to be inferior to DOAC and vitamin K antagonists and most likely superior to placebo. It can be hypothesized that in patients on aspirin, although the overall recurrence rate is higher than in patients on DOACS or vitamin K antagonists, emboli, potentially leading to the death of a patient, are either less frequent and/or smaller. When the composite endpoint of fatal bleeding and fatal recurrent VTE was evaluated, aspirin was, as expected, superior to placebo and even, rather unexpected, the rate was lower than in patients on DOACs or Vitamin K antagonists. Of course it has to be kept in mind that the number of these events is low, that the studies were not powered for these outcomes, and that there was no direct comparison with all three treatment options.

Although these data support the use of aspirin in patients with unprovoked VTE who terminated initial anticoagulant treatment, there are so far no final data directly comparing aspirin with anticoagulants. It is well known that anticoagulatory drugs reduce risk of recurrent VTE by more than 80 % (Kearon et al. 2012), which is superior to the risk reduction observed in aspirin users, and new oral anticoagulants, which do not need regular blood controls and dose adjustment, have made anticoagulant treatment nowadays more convenient compared to the use of vitamin K antagonists. More data on the use of either aspirin or new direct oral anticoagulants for the prevention of recurrent VTE will derive from a currently ongoing study, which compares the direct oral anticoagulant rivaroxaban in two doses to aspirin 100 mg for the extended treatment of VTE (EINSTEIN CHOICE). This study plans to include a total of 2850 patients (Weitz et al. 2015).

Conclusion

In conclusion, platelets play an important role in VTE. Experimental studies demonstrate the involvement of platelets in the formation of venous thrombi. Platelets release soluble proteins, which may be used as biomarkers to improve risk prediction of VTE. sP-selectin is the most studied biomarker and was shown to predict risk of recurrent VTE after a first event and risk of cancer-associated VTE. Finally, clinical studies have proven that platelets are a therapeutic target for primary and secondary prophylaxis of VTE. The use of aspirin is relatively safe, cheap, and widely available and has proven to be effective for prevention of VTE in different settings. A study that directly compares aspirin with the direct oral anticoagulant rivaroxaban for extended thromboprophylaxis after VTE is currently ongoing and will soon provide more information on the risks and benefits of either aspirin or anticoagulants in long-term secondary prevention of VTE.

Take-Home Messages

- Experimental studies provide evidence for an important role of platelets in the formation of venous thrombi.
- Platelet biomarkers, such as soluble P-selectin, are elevated in patients with acute VTE and predict risk of recurrent VTE and cancer-associated VTE.
- Although anticoagulant drugs are the first line of therapy used for reducing risk of VTE after orthopedic surgery and risk of recurrent VTE after a first event, also antiplatelet therapy with aspirin has been shown to be effective in these indications and might provide an additional therapeutic option.

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Gene Regulation of Platelet Function

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Abstract

Normal function of blood platelets is a complex multistep process that is crucial for maintenance of hemostasis and wound healing. Abnormality of platelet function may cause bleeding or thrombosis and may result in death. Platelet function is regulated by many genes, miRNAs, mRNAs, and proteins at different phases of megakaryocyte maturation and thrombopoiesis. Information on genes that regulate platelet function is obtained from studies of blood disorders using a candidate gene approach, a functional approach, or by using genome-wide association studies. Recent advances in DNA sequencing technologies and in meta-analysis of genome-wide association studies data and epigenomic, transcriptomic, and functional information have allowed identification of novel genes and noncoding genomic DNA regions that play an important role in platelet formation and function that affect thrombotic risk. This chapter is focused on genes that are known to regulate main platelet functions and on novel approaches to identify and characterize platelet-related genes.

Abbreviations

AHC	Alternating hemiplegia of childhood
ET	Essential thrombocythemia
GPS	Gray platelet syndrome
GWAS	Genome-wide association study
HPS	Hermansky–Pudlak syndrome
MK	Megakaryocytes
MPV	Mean platelet volume
P	*p*-value
PBMC	Peripheral blood mononuclear cells
PC	Platelet count
PCR	Polymerase chain reaction
RNAseq	RNA sequencing
RT	Reactive thrombocytosis
SAGE	Serial analysis of gene expression
VWF	von Willebrand factor
WT	Wild-type control subjects

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Introduction

Platelets are anucleate circulating peripheral blood cells that are crucial for basic biologic processes such as maintenance of normal hemostasis, inflammation, and wound healing. Platelets retain megakaryocyte-derived mRNAs (Gnatenko et al. 2003; Bugert et al. 2003; Bray et al. 2013; Rowley et al. 2011) and have evolved unique adaptive signals for maintenance of genetic and protein diversity (Denis et al. 2005; Weyrich et al. 1998) (Fig. 1). Quiescent platelets generally display minimal translational activity, although maximally activated platelets retain the capacity for protein synthesis, with implications for modulating arthritis-associated inflammation (Boilard et al. 2010) or the production of platelet progeny in vivo (Schwertz et al. 2010). Platelets are characterized by mean platelet volume and count, which vary from one individual to another, and platelet functions such as aggregation, adhesion, signaling, storage and secretion of content of α and dense granules, and others. Platelets that exhibit functional extremes convey a commensurate increased risk for thrombosis or bleeding. Interestingly, the propensity for such extremes has been shown to be heritable (Jones et al. 2007; O'Donnell et al. 2001; Bray et al. 2007;

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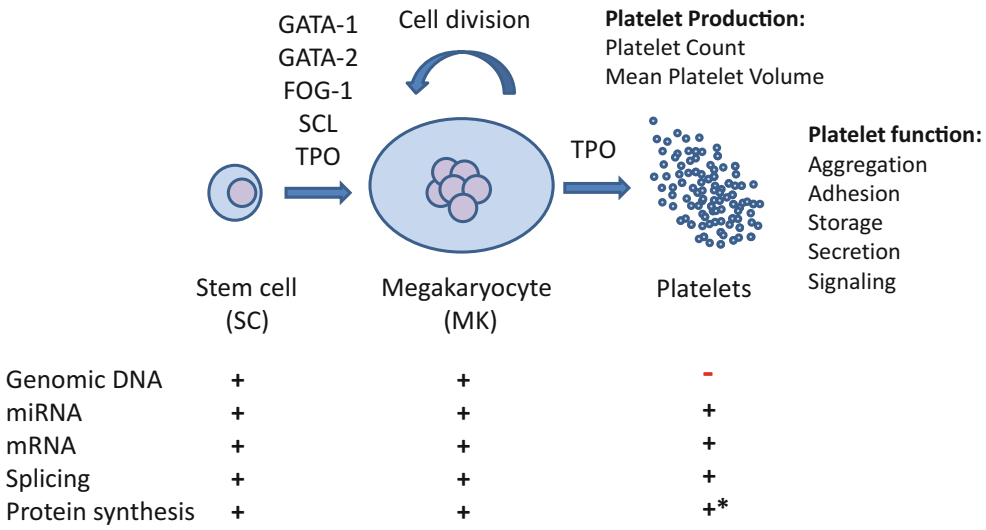

Fig. 1 Simplified schema of platelet production and function. Stem cells differentiate toward megakaryocytes (MK), and platelets are generated from MK as a result of thrombopoiesis. Platelets retain MK-derived miRNA, mRNAs, and proteins but lack genomic DNA. Selection of mRNAs for packaging into platelets is an active process, so transcriptomes of MK and platelets are significantly different. Platelets also retain competent pathways capable of converting precursor miRNAs into mature miRNAs and splice pre-mRNA into mRNAs. *Asterisk* circulating platelets generally display minimal translational activity, although they are capable for protein biosynthesis upon activation

Westbury et al. 2015), and accumulating evidence has established the critical genetic regulation of platelet function, using both genomic and transcriptomic approaches. Although recent advancements in genomics and bioinformatics have allowed identification and characterization of genes and noncoding regions of the human genome that are involved in regulation of platelet function, an understanding of underlying processes remains limited.

Platelet function is a complex multistep process with the potential for genetic regulation at multiple stages of activation. Once a blood vessel is damaged, platelets are exposed to the components of the extracellular matrix, particularly to collagen. This interaction triggers a long chain of receptor–ligand interactions that results in platelet activation and ultimately leads to the formation of a stable platelet plug or thrombus. This process consists of at least three phases (initiation, extension, and consolidation), each of which is achieved through a tightly regulated coordination of distinct receptor/ligands interactions. Thus, many candidate gene approaches apply these well-characterized interactions for gene/SNP-hunting strategies. For example, the initiation phase begins when plasma von Willebrand factor (VWF) binds to collagen via its A3 domain, resulting in structural change. The A1 domain of altered VWF binds to the platelet membrane receptor glycoprotein Ib-IX-V complex (GPIb complex). Then GPIbα or GPIb makes direct contact with VWF. This interaction leads to the adhesion of platelets to exposed thrombogenic surfaces such as sites of vessel wall injury. In parallel, the platelet-specific receptor glycoprotein VI (GPVI) and platelet integrin $\alpha_2\beta_1$ are involved in the formation of the platelet monolayer on the collagen surface.

During the extension phase, more platelet proteins come into action. Most of the receptors engaged in the extension phase are members of the G protein-coupled receptor family. Signaling from these receptors triggers conversion of prothrombin to thrombin at the membrane of activated platelets and release of active compounds from platelet α-granules and δ-granules. During extension phase, activated platelets release agonists that trigger a cascade of events leading to the activation of additional platelets in closest proximity to the site of vessel injury. Activated platelets secrete the contents of their α-granules and δ-granules into the surrounding blood to induce secondary stimulation and to activate other platelets. In the consolidation phase, platelet-to-platelet aggregation is mediated by the binding of fibrinogen and VWF to the activated platelet integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb-IIIa). This results in assembly of a fibrin network and generation of platelet aggregates (thrombus). Nonetheless, candidate gene approaches are limited, and open architectural systems designed for more robust (and unbiased) gene discovery remain applicable for the dissection of platelet genes regulating function and disease susceptibility. In this chapter we will review the genetic regulation of platelet function focusing on geno/phenotypic data modulating thrombotic risk. Novel methodologies focusing on transcriptomic and biomarker identification in platelet-associated thrombotic risk will also be reviewed.

Unraveling Genetic Regulation of Platelet Function

To date, considerable information has been generated on genes that regulate platelet function and affect risk of thrombosis. These genes encode for platelet membrane proteins, cytoskeleton proteins, kinases, proteins involved in cellular

trafficking, transcription factors, proteins of signal transduction pathways, proteins involved in megakaryocyte development, and others. Information on genes that play important roles in regulation of platelet function comes from several types of studies. The first type includes studies of various platelet diseases (inherited or acquired) associated with functional abnormalities and has resulted in identification and characterization of many of the receptor/ligand interactions modulating key platelet functions. The second type of studies uses candidate gene approach. In the past these studies focused mainly on genes that were considered to be important in platelet function, typically platelet receptors and signaling proteins. This approach requires large cohorts of subjects to detect rare variants and to elucidate their effects with sufficient statistic power. More promising approach is functional genomics that allows characterization of a larger number of potential gene candidates. This group of studies includes large-scale genome-wide association studies (GWAS) and meta-analyses of multiple sets of data. These studies represent an examination of many common genetic variants in different individuals to see if any variant is associated with a trait, focusing mostly on associations between single-nucleotide polymorphisms (SNPs) and certain phenotype. GWASs allowed characterization of coding and noncoding regions of human genome that contribute to normal platelet function. More recently, a novel approach has been developed that combines several independent GWAS into one large meta-analysis. Although results of such meta-analyses need thorough validation, this approach represents the most powerful tool to date to identify and characterize genes and genomic loci that play important role in thrombopoiesis and platelet function.

Interestingly, information on genes that regulate platelet function comes also from studies of other diseases where platelet abnormalities have been observed. Thus, based on the presence of secretory organelles, platelets are thought to share some morphological and functional features with neurosecretory cells (Pletscher 1986). Using platelets as model cells to study neuropathology is a concept that has been utilized since the early 1960s (Goubau et al. 2014). Significant variability of platelet characteristics such as aggregation and dense granule secretion (Yao et al. 1994), monoamine oxidase and platelet phospholipase A2 activity (Marcolin and Davis 1992; Gattaz et al. 1995), serotonin metabolism (Yao et al. 1996), and others (Kaiya et al. 1990) has been documented for patients with schizophrenia. Many of these platelet characteristics, including serotonin (Borgdorff and Tangelder 2012) and monoamine oxidase activity (Mosnaim et al. 1990), play a role in migraine exacerbations. Furthermore, variability of platelet characteristics has been reported for patients with Parkinson disease [altered monoamine oxidase type B activity (Finch et al. 1995; Husain et al. 2009)], autism [decreased platelet activity (Safai-Kutti et al. 1985) and platelet hyperserotonemia (Anderson et al. 2002; Hranilovic et al. 2009)], and Alzheimer disease [expression level and metabolism of amyloid precursor protein (Davies et al. 1997; Jelic et al. 2013) and elevated level of activated platelets (Casoli et al. 2013)].

Platelet Receptors and Thrombohemorrhagic Risk

Platelet receptors play a crucial role in platelet function. Among them are receptors involved (i) in platelet adhesion via glycoprotein (GP)VI (Trifiro et al. 2009), $\alpha_2\beta_1$ integrin (Ajzenberg et al. 2005), or VWF-GP1bα (Afshar-Kharghan et al. 1999), (ii) in fibrinogen-dependent platelet aggregation via $\alpha_{IIb}\beta_3$ integrin (Michelson et al. 2000), or (iii) in platelet signaling (Fontana et al. 2006; Hetherington et al. 2005). It was demonstrated that abnormalities of genes that encode platelet receptors severely affect platelet function (Table 1). Two platelet receptors play key role in collagen binding that initiates platelet activation—the integrin $\alpha_2\beta_1$ and the platelet-specific receptor GPVI. Polymorphism in these receptors results in altered receptor expression (Kunicki et al. 1997) and increased risk of acute myocardial infarction (Joutsi-Korhonen et al. 2003). ADP receptors $P2Y_1$ and $P2Y_{12}$ play major role in ADP-mediated platelet aggregation. Different polymorphic variants have been reported in the genetic region encoding for both genes located on chromosome 3 (Cavallari et al. 2007; Fontana et al. 2003). A study focused on 97 candidate genes involved in collagen and adenosine diphosphate (ADP) signaling pathways characterized 17 novel independently associated SNPs (from total 1327 SNPs) in 500 healthy Northern European subjects (Jones et al. 2009). Some of them were associated with increased risk of coronary and peripheral artery disease. Interestingly, polymorphism at *rs*1472122, located in this genetic region on chromosome 3, was associated with platelet function (Jones et al. 2009).

Studies of genetic basis of Bernard–Soulier syndrome (characterized by a prolonged bleeding time, thrombocytopenia, and extremely large platelets) demonstrated involvement of four separate genes—*GPIBA*, *GPIBB*, *GP9*, and *GP5* (Salles et al. 2008; Bolton-Maggs et al. 2006). These genes encode platelet-specific receptors and mutations affect binding of VWF, P-selectin, and other agonists, severely altering normal platelet function. Studies of platelet-related bleeding disorders, such as Glanzmann thrombasthenia, caused by mutations in integrins α_{IIb} [glycoprotein (GP) IIb] or β_3 (GP IIIa), have provided important insight into genes that regulate platelet function (Nurden and Nurden 2011; Lecine et al. 1998). Scott syndrome is a rare inherited disorder caused by defective scrambling of phospholipids in blood cells including platelets (Salles

Table 1 Genes encoding receptors associated with essential platelet functions

No.	Gene symbol	Protein	Affected platelet function	Notes	References
1	*GP1BA* *GP1BB* *GP6* *GP9*	Glycoproteins, IbA and IbB, glycoprotein VI Glycoprotein IX	Impaired platelet production and adhesion to vWF	Bernard–Soulier syndrome	Bolton-Maggs et al. (2006), Nakamura et al. (2006), Salles et al. (2008) and Poujol et al. (2002)
2	*ITGA2*	Integrin, alpha 2	Interaction with collagen	Bleeding disorder platelet-type 9	Jacquelin et al. (2001) and Kunicki and Nugent (2010)
3	*ITGA2B* *ITGB3*	Integrin, alpha 2b Integrin, beta 3	Platelet production and aggregation	Glanzmann thrombasthenia	Nurden and Nurden (2015), Nurden (2006), Blue et al. (2010), Peretz et al. (2006), Fiore et al. (2010) and Nurden et al. (2013)
4	*P2RY12*	Purinergic receptor P2Y, G protein coupled, 12	ADP-induced aggregation/ signaling	Bleeding disorder, platelet-type 8	Hollopeter et al. (2001), Cattaneo (2011), Watson et al. (2010) and Jones et al. (2009)
5	*P2RX1*	Purinergic receptor P2X, ligand-gated ion channel, 1	Decreased ATP-dependent platelet response	Bleeding disorder due to P2RX1 defect, somatic	Oury et al. (2000)
6	*TBXA2R*	Thromboxane A2 receptor	Reduced arachidonic acid-induced ATP secretion	Bleeding disorder, platelet-type 13	Mumford et al. (2010)
7	*F2R*	Coagulation factor II (thrombin) receptor	Thrombin-mediated platelet activation	Thrombosis, metastases in melanoma	Dupont et al. (2003) and Hoek (2007)
8	*ADRA2A*	Adrenoceptor alpha 2A	Shear-mediated platelet activation	Systolic heart failure, attention-deficit hyperactivity disorder	Yabe et al. (2006)
9	*FCGR2A*	Fc fragment of immunoglobulin gamma IIa receptor (antigen CD32)	Reduced platelet activation	Cystic fibrosis, thrombocytopenia	van der Pol and van de Winkel (1998)
10	*PEAR1*	Platelet endothelial aggregation receptor 1	Decreased platelet response to agonists	No known phenotype	Jones et al. (2009) and Faraday et al. (2011)

et al. 2008). This results in decreased fibrin formation at site of vascular injury due to lack of thrombin generation and phosphatidylserine expression (Morel et al. 2011). It was demonstrated that heterozygous missense mutation in ATP-binding cassette transporter A1 (gene *ABCA1*) (Albrecht et al. 2005) and mutation at the splice-acceptor site of the gene *TMEM16F* that causes premature termination of encoded transmembrane protein 16F (Suzuki et al. 2010) play a significant role in this disease.

SNPs in gene *PEAR1* (encoding platelet endothelial aggregation receptor) are associated with increased platelet response to agonists and increased PEAR1 protein expression after platelet degranulation. Later, sequencing confirmed that variation at *rs*12041331 accounted most strongly ($P = 2.07 \times 10^{-6}$) for the relation between the *PEAR1* gene and platelet function phenotype (increased platelet aggregability) (Faraday et al. 2011).

Many platelet stimulatory agonists mediate signaling via G proteins by binding to G protein-coupled receptors (Van Geet et al. 2009). The alpha subunit of the stimulatory G protein (GSα) regulates level of cyclic adenosine monophosphatase in cells. GSα is encoded in GNAS, an imprinted region on chromosome 20q13 that gives rise to several transcripts, antisense transcripts, and noncoding RNAs. The complexity of the GNAS cluster results in ubiquitous genomic imprints, tissue-specific GSα expression, and multiple genotype–phenotype relationships. Phenotypes associated with genetic and epigenetic abnormalities of the GNAS region include Albright's hereditary osteodystrophy (AHO), pseudohypoparathyroidism types Ia (PHPIa) and Ib (PHPIb), and pseudopseudohypoparathyroidism (PPHP). A variable degree of platelet GSα hypofunction was demonstrated in these patients (Freson et al. 2008).

Non-receptor Platelet Proteins and Thrombohemorrhagic Risk

Many other proteins are known to play significant role in platelet function. Among them are transcription factors, kinases, cytoskeletal proteins, proteins involved in signal transduction, and others (Table 2). It was demonstrated that several transcription factors play important roles during magakaryopoiesis that affect thrombopoiesis and platelet function. Thus, mice lacking transcription factor NFE2 have a late arrest in megakaryocyte maturation, resulting in profound thrombocytopenia (Lecine et al. 1998). Forkhead box protein 3 (*FOXP3* gene) is another transcription factor,

Table 2 Non-receptor platelet genes that regulate platelet function

No.	Gene symbol	Protein	Affected platelet function	Notes	References
1	*JAK2*	JAK 2 tyrosine kinase	Complex signaling pathways, platelet production	Multiple myeloproliferative disorders	James et al. (2005), Lussana et al. (2009) and Scott (2011)
2	*ATP1A3*	ATPase alpha(III) subunit	Reduced platelet aggregation	Congenital Rett syndrome	Di Michele et al. (2013)
3	*FLNA*	Filamin A	Reduced platelet aggregation, signaling, secretion, adhesion to vWF	Thrombocytopenia	Nurden et al. (2011), Berrou et al. (2013) and Falet (2013)
4	*FOXP3*	Forkhead box P3	Megakaryopoiesis defects	Thrombocytopenia	Fontenot and Rudensky (2005) and Bernard et al. (2009)
5	*FOXG1*	Forkhead box G1	ATP secretion, epinephrine-induced aggregation	Congenital Rett syndrome variant	Goubau et al. (2013a)
6	*AQP7*	Aquaporin 7	ATP secretion, impaired aggregation, enlarged platelets	Hyperglyceroluria and a mild platelet secretion defect	Goubau et al. (2013b)
7	*VPS33B*	Vacuolar protein sorting 33 homolog B	Platelet aggregation, response to ADP	Arthrogryposis–renal dysfunction–cholestasis (ARC) syndrome	Lo et al. (2005)
8	*GNAS*	GNAS complex locus	GSα hypofunction, reduced cAMP formation	Pseudohypoparathyroidism Ib	Freson et al. (2008)
9	*GNAZ*	Guanine nucleotide-binding protein (G protein), alpha z polypeptide	cAMP-dependent platelet activation	No known phenotype	Jones et al. (2009)
10	*TBXAS1*	Thromboxane A synthase 1	Reduced arachidonic acid-induced aggregation	Ghosal syndrome	Genevieve et al. (2008)
11	*RUNX1*	Runt-related transcription factor 1	Broad defects of platelet function	Acute myeloid leukemia	Song et al. (1999) and Sun et al. (2007)
12	*WAS*	Effector protein for Rho-type GTPases	Decreased platelet aggregation, small platelets, decreased count	Wiskott–Aldrich syndrome	Burns et al. (2004), Blundell et al. (2010), Gulacsy et al. (2011) and Villa et al. (1995)
13	*MYH9*	Myosin, heavy chain 9, non-muscle	Decreased platelet production, presence of giant platelets	May–Hegglin anomaly and Fechtner and Sebastian syndromes	Seri et al. (2000) and Kunishima and Saito (2010)
14	*MLPH*	Melanophilin	Abnormal δ-granules	Griscelli syndrome	Huizing et al. (2008)
15	*RAB27A*	RAB27A, member RAS oncogene family	Reduced aggregation	Griscelli syndrome	Menasche et al. (2000)
16	*GATA1*	GATA-binding protein 1 (globin transcription factor 1)	Reduces collagen-induced aggregation, enlarged platelets	Anemia, x-linked, with/without neutropenia and/or platelet abnormalities	Hughan et al. (2005) and Tubman et al. (2007)
17	*NFE2*	Nuclear transcription factor, erythroid 2	Arrest in late stage of megakaryocyte maturation	Thrombocytopenia	Lecine et al. (1998)
18	*FYB*	FYN-binding protein	Reduced platelet activation	Congenital autosomal recessive small-platelet thrombocytopenia	Levin et al. (2015)
19	*ABCA1*	ATP-binding cassette, subfamily A (ABC1), member 1	Failure to expose phosphatidylserine to the outer leaflet of the platelet plasma membrane	Scott syndrome	Albrecht et al. (2005)
20	*ANO6* (*TMEM16F*)	Transmembrane protein 16F	Phosphatidylserine signaling in platelets	Scott syndrome	Suzuki et al. (2010)
21	*NBEAL2*	Neurobeachin-like protein 2	Defects of α-granules formation	Gray platelet syndrome	Albers et al. (2011)
22	*HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *HPS6*, *DTNBP1* and *PLDN*	Various proteins	Dense granule defects	Hermansky–Pudlak syndrome	Cullinane et al. (2011) and Gunay-Aygun et al. (2004)
23	*PTGS1*	Cyclooxygenase 1 (COX-1)	Platelet response to aspirin, platelet aggregation	Resistance to aspirin	Maree et al. (2005)

which is believed to be restricted to a subset of regulatory T cells and is required for their development (Fontenot and Rudensky 2005). Humans with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) and the scurfy [Foxp3(sf)] mouse have mutations in the *FOXP3* gene that lead to a host of pathologies including autoimmunity and skin diseases. Interestingly, scurfy mice and some humans with IPEX are also thrombocytopenic. Human and mouse megakaryocytes express *FOXP3* mRNA and protein. Recently it was demonstrated that *foxp3*-deficient mice and human megakaryocyte progenitors exhibit proliferation defects (Bernard et al. 2009). Striking platelet abnormalities were observed in both an IPEX patient and *foxp3*(sf) mice. Impaired platelet spreading and release of TGF-beta and CD40 ligand (CD40L) were repeated. *Foxp3*(sf) mice were thrombocytopenic and had increased platelet volume and altered serum levels of CD40L, TXB(2), and TGF-beta. It was demonstrated that mutations or reduced expression of another transcription factor and a member of forkhead box proteins, forkhead box G1 (*FOXG1*), results in abnormal platelet production (Goubau et al. 2013a).

Megakaryocyte maturation is a complex process that includes proplatelet formation—rearrangement of the cytoskeleton that results in formation of long cytoplasmic extensions (Thon et al. 2010). These extensions are needed to transport platelet granules and organelles to forming platelets. Cytoskeletal proteins play crucial role in this process. Actin-binding protein filamin A is a 280 kD protein that cross-links actin filaments into orthogonal networks in cortical cytoplasm and participates in the anchoring of membrane proteins for the actin cytoskeleton. Filamin A, encoded by the *FLNA* gene, is a widely expressed protein that regulates reorganization of the actin cytoskeleton by interacting with integrins, transmembrane receptor complexes, and second messengers. Filamin A is also a predominant filamin isoform expressed in platelets. In platelets, FLNA tethers the principal receptors ensuring the platelet–vessel wall interaction, end binding of glycoprotein Ibα and integrin $\alpha_{IIb}\beta_3$, to the underlying cytoskeleton. Patients with filaminopathy A often have defective platelet adhesive functions and thrombocytopenia (Nurden et al. 2011; Berrou et al. 2013; Falet 2013).

Studies of other diseases, not directly related to platelets, often contribute to our understanding of genetic regulation of platelet function. Alternating hemiplegia of childhood (AHC) is a rare syndrome with repeated hemiplegic episodes, paroxysmal events, and global neurological impairment. Recently, exome sequencing by two independent groups revealed de novo missense mutations in the *ATP1A3* gene (Palmgren and Nissen 2011), coding for the isoform α3 of the sodium/potassium ion channel that maintains ion gradients. Platelets from AHC patients presented with structural and functional abnormalities of granules positive for the lysosomal marker CD63 (Di Michele et al. 2013). Though AHC platelets have a normal number of dense granules with a normal total ATP content, they present an abnormal dense core structure. Therefore, the mild platelet activation after epinephrine stimulation which results in a delayed release of dense granules can be due to the abnormal granule reorganization before release, such as observed after platelet spreading on fibrinogen. Indeed granules positive for the lysosomal marker CD63 (including dense granules) in AHC cases seem to fuse and centralize more than in control platelets before secretion.

Recently, studies of a family of five children affected with a novel autosomal recessive bleeding disorder with small-platelet thrombocytopenia identified a homozygous deleterious nonsense mutation 393G>A in *FYB* gene. This gene encodes adhesion and degranulation-promoting adaptor protein (ADAP), a hematopoietic-specific protein involved in platelet activation, cell motility and proliferation, and integrin-mediated cell adhesion. Platelets from such patients showed increased basal expression of P-selectin and PAC-1, and reduced increments of activation markers after stimulation with ADP, as detected by flow cytometry; they also showed reduced pseudopodium formation and the presence of trapped platelets between the fibrin fibers after thrombin addition, as observed on scanning electron microscopy (Levin et al. 2015).

Aquaporin 7 (*AQP7*) belongs to the aquaglyceroporin family, proteins that transport glycerol and water. AQP7-deficient mice develop obesity, insulin resistance, and hyperglyceroluria. Homozygous mutation of AQP7 (G264V) (Goubau et al. 2013b) has been reported as a possible cause of psychomotor retardation and hyperglyceroluria in three unrelated children. Platelets from these children demonstrated reduced ATP secretion and an absence of a secondary aggregation wave after epinephrine stimulation. Electron microscopy revealed round platelets with centrally located granules. Vascular protein sorting-associated protein 33B is encoded by *VPS33B* gene in human genome. Recent study demonstrated that this protein is essential for alpha granule biogenesis and patients have enlarged platelets with strongly reduced number of alpha granules and reduced aggregation response (Lo et al. 2005). An important, but poorly characterized, role of signaling through integrins and receptor tyrosine kinases adds further complexity to the final phase of clot formation. Current research indicates an essential role for outside-in signaling through integrins and via receptor tyrosine kinases, including members of the Eph kinase family (Kunicki and Nugent 2010).

Several epidemiological and twin studies suggest that the extent of platelet aggregability may be heritable (Rissanen

and Nikkila 1979; Berg 1981; Burton et al. 2007; Nikpay et al. 2015). Although definitions for platelet responsiveness tend to differ among studies, it is now widely accepted that platelet aggregation ex vivo in response to agonist stimulation varies considerably among healthy individuals. In an analysis of 359 healthy people, it was demonstrated that a minority consistently showed hyperresponsiveness (≥65 % maximal platelet aggregation) after stimulation with ADP, collagen, epinephrine, collagen-related peptide, or ristocetin (Yee et al. 2005). Female gender and higher fibrinogen levels were significantly associated with hyperresponsiveness, and hyperreactivity to one agonist tended to persist with others in the assays studied. Recently, GWAS study of 2.5 million SNPs with platelet aggregation responses to three agonists (ADP, epinephrine, and collagen) identified associations of seven loci with platelet aggregation near or within *GP6*, *PEAR1*, *ADRA2A*, *PIK3CG*, *JMJD1C*, *MRVI1*, and *SHH*. Six of these loci replicated at $P < 0.05$ in an additional cohort (Johnson et al. 2010).

Gray platelet syndrome (GPS) is a predominantly recessive platelet disorder that is characterized by mild thrombocytopenia with large platelets and a paucity of α-granules, frequently associated with moderate bleeding. Recently, exome sequencing identified gene NBEAL2 as the causative gene for this disease (Albers et al. 2011). It encodes for *neurobeachin*-like protein 2 that has no known function but is a member of a gene family that is involved in granule development. Silencing of nbeal2 in zebrafish abrogated thrombocyte formation.

Similar to defects of α-granules, abnormalities of δ-granules severely affect platelet function. The bulk of our knowledge concerning dense granule deficiency disorders derives from studies of Hermansky–Pudlak syndrome (HPS), a rare disorder associated with oculocutaneous albinism (decreased pigmentation) and bleeding problems due to a platelet abnormality (platelet storage pool defect). To date, there are nine known genes and genomic loci that cause HPS (Gunay-Aygun et al. 2004; Cullinane et al. 2011). These genes include *HPS1*, *AP3B1*, *HPS3*, *HPS4*, *HPS5*, *HPS6*, *DTNBP1*, and *PLDN*.

Aspirin, nonsteroidal anti-inflammatory drugs like indomethacin, and other drugs severely affect platelet function. Platelet response to these drugs is regulated at the gene level. Thus, it was demonstrated that polymorphism in platelet receptors $P2Y_1$, $P2Y_{12}$, and ITGB3 is associated with an aspirin-resistance phenotype (Jefferson et al. 2005; Lev et al. 2007). Genetic variability in *PTGS1* gene, encoding for platelet cyclooxygenase COX-1 (an enzyme involved in the conversion of arachidonic acid to the potent platelet agonist thromboxane), is associated with platelet response to aspirin and platelet aggregation (Maree et al. 2005). Despite recent progress, our understanding of genetic regulation of platelet responses to drugs remains limited. Future studies will certainly identify more genes and genomic loci that regulate platelet–drug interactions. This information will ultimately lead to a better understanding of platelet function and result in more personalized use of platelet-affecting drugs.

Mean Platelet Volume, Platelet Count, and Thrombotic Risk

Mean platelet volume (MPV) and platelet count (PC) are highly inheritable and affect platelet function, although they significantly vary from one individual to another. Recent studies identified multiple genes and noncoding regions in human genome that are associated with MPV and PC and demonstrated that they correlate with agonist-induced platelet function (Nurnberg et al. 2012; Kunicki et al. 2012; Huczek et al. 2005). Furthermore, MPV has been suggested as an independent cardiovascular risk factor (Slavka et al. 2011). MPV is also associated with megakaryopoiesis and beta-thromboglobulin release (Bath and Butterworth 1996). Whole genome expression analysis revealed highly significant association of the SNP *rs*342293 (located on chromosome 19) with MPV in healthy subjects (Soranzo et al. 2009; Meisinger et al. 2009; Shameer et al. 2014; Qayyum et al. 2012). Analysis of electronic medical records identified five chromosomal regions associated with PC and eight regions associated with mean platelet volume ($P < 5 \times 10^{-8}$) (Shameer et al. 2014). This study identified 20 SNPs that affect PC and 22 SNPs that affect MPV.

Recent advances of sequencing technology in conjunction with rapidly expanding knowledge of the human genome and development of modern bioinformatics approaches have made it possible to combine several GWAS studies into one meta-analysis. Such an approach represents a very powerful tool for identification of genes, genomic regions, and individual SNPs that play crucial role in platelet formation and function. The study of Gieger et al. remains the largest platelet-focused meta-analysis of GWAS so far (Gieger et al. 2011). In this study, analysis of 66,867 individuals of European ancestry identified 68 genomic loci that are reliably associated with platelet volume and establishing novel regulators of megakaryopoiesis and platelet production. Findings of this meta-analysis are independently validated using gene silencing in model organisms, stem cell differentiation, and analysis of transcriptomic information. Later, results of this mega-analysis have been validated in cohorts with different ethnic backgrounds (Lo et al. 2011; Qayyum et al. 2012).

Using 13 independent studies related to mean platelet volume (18,600 individuals totally) and 23 studies related to platelet count (48,666 individuals totally), the authors performed meta-analysis linking these two parameters to

~2.5 million mapped SNPs (Gieger et al. 2011). Initially, association between these parameters and each SNP was tested using an additive model within each study. Then, study-specific test statistics was combined into a fixed-effect meta-analysis, and the level of spurious associations was reduced. Overall, 68 independent genomic regions were identified that were associated with mean platelet volume and platelet count [p-value (P) $< 5 \times 10^{-8}$], of which 52 were new and 16 have been previously characterized in Europeans. Of these, 43 loci were associated with platelet count and 25 with platelet volume. To validate these results, these associations were tested in subjects of south Asian and Japanese origin, demonstrating significant similarity.

A large proportion of these genomic loci (46 out of 68) were associated with SNPs located in gene-coding regions of human genome, including several key genes—regulators of hemostasis (*ITG2AB, F2R, GP1BA*), megakaryopoiesis (*THPO, MEF2C*), and platelet lifespan (*BAK1*). During further narrowing of the analysis, 54 "core" genes were selected as either containing a SNP or located within 10 kb from a SNP associated with platelet volume or count. Ingenuity pathway analysis demonstrated that these genes demonstrate significant overrepresentation in biological pathways such as hematological disease, cancer, or regulation of cell cycle.

Interestingly, core genes tend to have significantly higher expression in megakaryocytes than in other blood cell types. To confirm this, umbilical cord blood-derived hematopoietic stem cells were differentiated toward precursors of blood cells, and genome-wide expression microarrays were used to study levels of gene expression. Core genes showed significant increase of expression over time in megakaryocytes, but not in erythroblasts ($P = 1.5 \times 10^{-6}$ and 0.77, respectively) (Gieger et al. 2011), demonstrating that these genes indeed play an important role in megakaryopoiesis and platelet formation.

To further evaluate biological role of these genes in megakaryopoiesis and platelets, 15 of core genes were silenced in *D. rerio* or *D. melanogaster* (Table 3). Silencing of four genes in *D. rerio* (orthologues of *AK3*, *JMJD1C*, *RNF145*, and *ARHGEF3*) resulted in the ablation of both primitive erythropoiesis and thrombopoiesis. Silencing of the orthologue of *TPM1* gene (tropomyosin 1 α) abolished formation of platelets, but not erythrocytes. For only one core gene, *EHD3*, silencing did not reveal any hematopoietic phenotype, even though two platelet-associated SNPs are located inside noncoding regions of this gene. Interestingly, silencing of gene *AK3*, located at 2699 kb from platelet-associated SNP, resulted in ablation of erythropoiesis and thrombopoiesis in *D. rerio*. One possible explanation is involvement of the region around this SNP in regulation of expression of this gene. Silencing of four other core genes (orthologues of *DNM3*, *BRF1*, *PSMD13*, *ZFPM2*) in *D. melanogaster* resulted in significant and reproducible differences in the number of produced erythrocytes and platelets.

Taken together, these data demonstrate the efficiency of a gene identification approach using meta-analysis of GWAS data. This study not only identified novel genes involved in megakaryopoiesis and platelet formation and function. It also validated these findings on several levels, including transcriptome studies during differentiation of stem cells toward precursors of blood cells and gene silencing using model organisms *D. rerio* and *D. melanogaster*.

Platelet Transcriptome and Platelet Function

In addition to regulation on genomic level, platelet function is also regulated at transcription and translation levels. Accumulating evidences suggest that miRNAs play significant role in regulation of platelet transcriptome. It was demonstrated that platelets retain a competent miRNA pathway capable of converting precursor miRNAs through functional Dicer/Argonaute 2 (*Ago2*) complexes (Landry et al. 2009), with evidence that Ago2-miRNA-223 complexes specifically regulate expression of the functionally important platelet purinergic $P2Y_{12}$ adenosine diphosphate (ADP) receptor. Additional evidence suggests that miRNAs (miR28) can modulate expression of the c-*mpl* thrombopoietin (Tpo) platelet receptor (Girardot et al. 2010) and that miR96-mediated regulation of endobrevin/*VAMP8* (vesicle-associated membrane protein 8) affects human platelet functional responsiveness (Kondkar et al. 2010). The potential importance of a functionally competent miRNA pathway is further highlighted by the enrichment of platelet miRNAs compared with other hematopoietic cells, such as granulocytes and megakaryocytes (Landry et al. 2009). It was shown that distinct platelet phenotypes can be genetically classified using a restricted set of mRNA or miRNA biomarkers (Gnatenko et al. 2010; Xu et al. 2012).

Platelets retain megakaryocyte-derived miRNAs and mRNAs (Gnatenko et al. 2003; Bugert et al. 2003; Bray et al. 2013; Rowley et al. 2011) but do not contain genomic DNA. Platelets also possess a functional spliceosome, a complex that processes pre-mRNAs in the nuclei of other cell types (Denis et al. 2005). Spliceosome components are present in the cytoplasm of human megakaryocytes and in proplatelets that extend from megakaryocytes. Primary human platelets contain essential spliceosome factors including small nuclear RNAs, splicing proteins, and endogenous pre-mRNAs. In response to integrin engagement and surface receptor activation, platelets precisely excise introns

Table 3 Core genes identified using metadata analysis (Gieger et al. 2011) of GWAS studies and validated in platelet formation using model organisms *D. rerio* and *D. melanogaster*

SNP (trait)	Core gene (distance from SNP, kb)	Protein	Phenotype in model organism
*rs*1094144 (MPV, PLC)	*DNM3* (0)	Dynamin 3	Overproliferation of plasmatocytes
*rs*1354034 (MPV, PLC)	*ARHGEF3* (0)	Rho guanine nucleotide exchange factor 3	Profound effect on thrombopoiesis and megakaryopoiesis
*rs*10076782 (MPV)	*RFN145* (0)	Ring finger protein 145	Ablation of thrombopoiesis and erythropoiesis
*rs*7075195, *rs*10761731 (MPV, PLC)	*JMJD1C* (0)	Jumonji domain containing 1C	Ablation of thrombopoiesis and erythropoiesis
*rs*3000073 (MPV)	*BRF1* (0)	RNA polymerase III transcription initiation factor 90 KDa subunit	Reduction in plasmatocyte number
*rs*505404, *rs*17655730 (MPV, PLC)	*PSMD13* (0)	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	Reduction in plasmatocyte number
*rs*6993770 (PLC)	*ZFPM2* (0)	Zinc finger protein, FOG family member 2	Reduction in plasmatocytes and crystal cells
*rs*3809566 (PLC)	*TPM1* (1115)	Tropomyosin 1, alpha	Total abrogation of thrombopoiesis, but normal erythropoiesis
*rs*409801 (PLC)	*AK3* (2699)	Adenylate kinase 3	Ablation of thrombopoiesis and erythropoiesis
*rs*708382 (PLC)	*ITGA2B* (7207)	Integrin, alpha 2b (platelet glycoprotein IIb of IIb/IIIa complex, antigen CD41)	Severely reduced thrombocyte function in *D. rerio*
*rs*649729, *rs*625132 (MPV, PLC)	*EHD3* (0)	EH domain containing 3	No hematopoietic phenotype

MPV mean platelet volume, *PLC* platelet count

from interleukin-1β pre-mRNA, yielding a mature message that is translated into protein. It was also shown that quiescent human platelets express pre-mRNA of tissue factor (coagulation factor III or thromboplastin, gene symbol *F3*), an essential cofactor for the activation of blood coagulation, and in response to activation, splice this intronic-rich message into mature mRNA (Schwertz et al. 2006).

Platelets have evolved unique adaptive signals for maintenance of genetic and protein diversity (Denis et al. 2005; Weyrich et al. 1998). Although the platelet transcriptome varies from one individual to another, more significant differences were demonstrated between platelet transcriptome of healthy subjects and patients with myeloproliferative disorder essential thrombocythemia (Gnatenko et al. 2003; Bugert et al. 2003; Bray et al. 2013; Rowley et al. 2011). This suggests potential use of mRNAs as biomarkers for diagnostics and prognostics of platelet disorders and also adds complexity to the understanding of genomic regulation of platelets function. Selection of mRNAs for packaging into platelets is an active process, as evident by significant difference between platelet and megakaryocyte transcriptomes (Cecchetti et al. 2011).

Initial studies, performed using microarray and serial analysis of gene expression (SAGE), provided first panoramic overview of human platelet transcriptome (Gnatenko et al. 2003; Bugert et al. 2003; McRedmond et al. 2004; Dittrich et al. 2006) and demonstrated that it includes 1500–3000 individual mRNA transcripts, some of which are platelet restricted. Recently, application of RNA sequencing technology (RNAseq) provided deeper view into RNA content of human platelets (Rowley et al. 2011; Bray et al. 2013). Comparison of human and mouse platelet transcriptomes revealed expression of 8582 human genes and 6012 mouse genes (RefSeq database), with 4990 genes overlap (<58 % overlap). In addition to mRNA transcripts, these studies revealed diverse classes of noncoding RNAs, including pervasive antisense transcripts to protein-coding loci, numerous abundant microRNAs, and thousands of novel unannotated long and short intronic transcripts (Bray et al. 2013). Efficient purification of platelets and separation of them from other blood cells, especially from leukocytes, are challenging. Considering size and RNA content of one platelet comparing to one leukocyte, even low-level contamination of platelet sample with leukocytes may significantly affect transcript profile. It is not excluded therefore that some low-abundant transcripts detected in platelets actually come from contaminating leukocytes. Nevertheless, platelet mRNA profiling information is consistent in literature.

In addition to polymorphism, variability of expression levels of key platelet proteins may affect platelet function. For example, expression level of the platelet integrin $\alpha_2\beta_1$ is highly variable in healthy subjects, and platelets expressing higher levels have an increased ability to bind collagen (Roest et al. 2000; Kunicki et al. 1993) and have elevated density of this receptor on platelet surface.

Platelet Transcriptomic Studies Designed to Evaluate Thrombotic Risk

The application of gene profiling has been used in clinical diseases associated with thrombosis using either platelets or peripheral blood mononuclear cells (PBMC) as the cellular source. Two studies have reported gene expression changes associated with ischemic stroke, differing in the use of PBMC (Moore et al. 2005) or neutrophils (Tang et al. 2006). The studies of platelet transcriptome revealed quantitative differences between acute and stable coronary artery disease (Healy et al. 2006). In particular, platelet mRNA profiling from patients with acute ST-segment-elevation myocardial infarction (STEMI, $n = 16$) or stable coronary artery disease ($n = 44$) identified 54 differentially expressed transcripts. The strongest discriminators of STEMI were *CD69* and myeloid-related protein-14 (MRP-14, gene symbol *S100A9*).

Essential thrombocythemia (ET) is a rare chronic blood disorder characterized by the overproduction of platelets by megakaryocytes in the bone marrow (Beer and Green 2009). It may, albeit rarely, develop into acute myeloid leukemia or myelofibrosis. The most common symptoms are bleeding, clotting, headache, nausea, vomiting, abdominal pain, visual disturbances, dizziness, fainting, and numbness in the extremities. Collectively, ET platelets demonstrated greater number of expressed transcripts in comparison with normal controls, although considerably less than the number of transcripts generally found in nucleated cells (Gnatenko et al. 2003, 2005). A class prediction model of thrombocytosis has been generated based on expression levels of 11 platelet-expressed mRNA biomarkers as studied by microarray and quantitative PCR (Gnatenko et al. 2010). In total, platelet transcript profiles of 126 subjects were studied by microarray—48 healthy control subjects (WT), 38 patients with reactive thrombocytosis (RT), and 40 patients with ET (24 of which contained the *JAK2*V617F mutation). Cross-platform consistency was validated using alternative transcript profiling method. Class prediction algorithms were developed to assign phenotypic class between the thrombocytosis cohorts and by *JAK2* genotype. Using microarray data, an 11-biomarker gene subset (*WASF3*, *CTNS*, *HIST1H2AG*, *ACOT7*, *LAPTM4B*, *TGFB2*, *TPM1*, *H3F3A*, *APP*, *NGFRAP1*, *CLEC1*) is discriminated among the three cohorts with 86.3 % accuracy, with 93.6 % accuracy in two-way class prediction (ET vs. RT). Subsequent quantitative PCR analysis established that these biomarkers were 87.1 % accurate in prospective classification of a second cohort. A 4-biomarker gene subset (*HIST1H1A*, *SRP72*, *C20orf103*, *CRYM)* predicted *JAK2* wild-type ET in more than 85 % patient samples using either microarray or quantitative PCR profiling, with lower predictive capacity in *JAK2* $V^{617}F$ mutant ET patients. These results establish that distinct genetic biomarker subsets can predict thrombocytosis class using routine phlebotomy. Furthermore, these results confirmed discriminant power of 11-biomarker subset in distinguishing ET from RT irrespectively of the platform technology used for transcript profiling. Although these genes are not known yet to play a direct role in platelet function, the variability of their expression levels is associated with abnormal platelet production and may increase thrombotic risk. Furthermore, transcript profiling of these mRNAs in circulating platelets using microarray, quantitative PCR, or fluorescent microbeads (Gnatenko et al. 2005, 2008, 2010; Huang et al. 2013) can be used for the diagnostics of ET, currently done by exclusion.

Conclusion

It is clear that recent progress in technology and in approaches to analyze human genome has resulted in considerable advances in our understanding of the genetic basis of platelet function and its contribution to thrombotic risk. As we illustrated in this chapter, meta-analyses of multiple genome-wide association studies in conjunction with traditional candidate gene approach and functional studies allowed identification of many genes and noncoding regions of human genome that affect platelet function. In the near future, when whole exome and genome sequencing will become a routine, we anticipate identification of novel genomic loci—candidates that define platelet function and affect thrombotic risk. To further understand genetic regulation of platelet function associated with risk of thrombosis, system biology approach is needed. Such approach would focus on studies of platelet functional responses as quantitative trait loci linked to overall platelet responsiveness. It would integrate genomic and transcriptomic information to variability of platelet activation and disease outcome (O'Donnell et al. 2001).

Take-Home Messages

- Genetic factors play a key role in substantial variation of platelet production and function seen among both healthy subjects and patients. To date, it is known that platelet function is regulated by many genes that encode various proteins. Among them are platelet receptors, transcription factors, kinases, cytoskeletal proteins, proteins involved in signal transduction, and others. Abnormalities of these genes often severely affect platelet function, resulting in disease or death.
- In addition, single-nucleotide polymorphism in certain noncoding regions of human genome is shown

(continued)

to affect platelet function. Despite significant progress in our understanding of human genome, little is known about molecular mechanisms of such effects.

- Platelet production and function are also regulated at transcription level. For example, studies of platelet transcriptome identified mRNA and miRNA biomarkers of essential thrombocythemia and revealed quantitative differences between acute and stable coronary artery disease that are associated with increased thrombotic risk.
- Rapidly expanding knowledge of human genome leads to identification of novel genes that affect platelet function. In the future, meta-analyses that combine multiple genome-wide association studies will identify more genes and genome regions that contribute to platelet formation and function.

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Part V

Pathology: Non Hemostatic or Thrombotic Disorders

Platelets and Bacterial Infections

Matthew T. Rondina, Olivier Garraud, and Hansjörg Schwertz

Abstract

Platelets are circulating blood cells classically known for their key roles in mediating hemostasis and vascular wall repair. Platelets also possess a dynamic repertoire of effector functions in the immune continuum that span from rapid innate immune responses to more delayed adaptive and acquired immune activities. Platelets express a wide array of structural and functional characteristics of host defense effector cells that augment host defenses to bacterial infections. Platelets display a diverse range of surface ligands and receptors that recognize and bind bacteria, including complement receptors, FcγRII, toll-like receptors (TLRs), and integrins conventionally described in the hemostatic response, such as $\alpha_{IIb}\beta_3$ and GPIb. Both direct and indirect binding of bacteria, bacterial toxins, and other agonists to platelets via fibrinogen, fibronectin, C1q, or von Willebrand factor (vWF) may result in platelet activation. Platelets may also internalize bacteria, although the function and fate of these host defense mechanisms remain incompletely understood. Once activated, platelets transform from quiescent discoid forms to amoeboid cells that chemotax to and target microbial pathogens or ligands displayed by tissues injured during infectious insults. Upon activation and subsequent degranulation, platelets secrete an array of multifunctional host defense and antimicrobial peptides that act as direct anti-infective agents and coordinate additional molecular and cellular host defenses. Platelets also release soluble immunomod-

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ulatory factors that play crucial roles in the formation of neutrophil extracellular traps (NETs), resulting in bacterial elimination but also enhancing thrombosis in disease situations. Therefore, platelets are increasingly recognized as key effector cells in immune and inflammatory responses to host infection. The multiplicity of events underscores the complexity of platelet–bacterial interaction and illustrates the emerging view that platelets are important sentinel and effector cells in host defenses against pathogens.

Introduction

Due to their high number in the circulation, platelets may be the first blood cell that encounters bacteria circulating systemically. As such, platelets have been suggested to be key sentinels residing within the vascular space. The amount and variety of functional immunoreceptors define these small cells as complex and unique blood "all-purpose" elements and weapons. Nevertheless, the recognition of platelets as fully functional immune cells is still controversial, primarily due to their lack of a nucleus and their structural "simplicity." In the last decade, a multitude of previously unrecognized and dynamic functions have been discovered for platelets. These emerging functions are comprehensive and adaptive and span the immune continuum. Many of these newly recognized functions are essential for pathogen surveillance, host defenses and containment, and wound healing (Hamzeh-Cognasse et al. 2015; Vieira-de-Abreu et al. 2012; Weyrich et al. 2003; Weyrich and Zimmerman 2004). In this chapter, we will discuss the role of platelets as innate and adaptive immune cells and the relationship of immune activities of platelets to their hemostatic effector functions.

We will first focus on platelet–bacterial interactions, platelet-released immunomodulatory molecules, and platelet-mediated host defense systems. We will also discuss the intersection of bacterial infection and thrombosis, the interactions of platelets with other classic immune cells, and the necessary cross talk between platelets and neutrophils in neutrophil extracellular trap (NET) formation.

Platelet–Bacterial Interactions: One-on-One

When talking about platelets and their potential to interact with bacteria, there are several different possible mechanisms of interaction (Fig. 1). First, bacteria themselves bind host plasma proteins, which have a

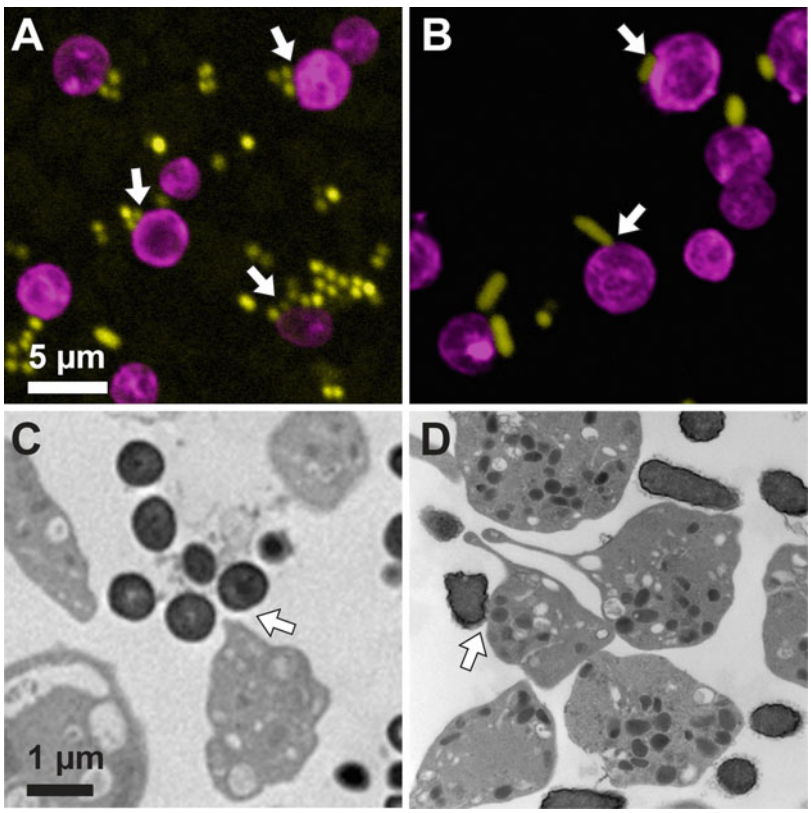

Fig. 1 Human platelets directly interact with bacteria. Human platelets were incubated with bacteria for 2 h. (**a**) Platelets were incubated with *S. aureus* or (**b**) *E. coli*, fixed, spun down, and subsequently stained using Topro-3 for DNA-containing bacteria (*yellow*) and wheat germ agglutinin (WGA, magenta). *White arrows* indicate sites of platelet–bacterial interaction. Platelets were analyzed using transmission electron microscopy (TEM) after being incubated with *S. aureus* or (**c**) *E. coli* (**d**). *White arrows* indicate sites of platelet–bacterial interaction

corresponding receptor on the platelet surface. Second is the direct binding or adhesion to platelet receptors or the platelet surface. Third is the interaction of secreted bacterial products, exotoxins, or bacterial degradation product with platelet surface receptors. Since platelets express a diverse array of surface receptors, these interactions are highly complex and may result in different activation patterns.

Indirect Interactions

The Integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa)

The platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ is a key mediator of the hemostatic function of platelets. The engagement of the integrin by fibrinogen leads to platelet adhesion, aggregation, and activation via outside-in signaling events and is a pharmaceutical target in several disease scenarios (Bennett et al. 1999, 2009; Coller 1997). One family of bacteria that provides an example for direct fibrinogen and fibronectin binding to platelets is the genus *Staphylococci*. Binding of *Staphylococci* to tissues and the extracellular matrix is a critical step in establishing infection of a host organism. To achieve this, *Staphylococci* express surface receptors belonging to the microbial surface components recognizing adhesive matrix molecules (MSCRAMM) family (Josefsson et al. 1998). Some of the most common examples of MSCRAMM are listed in Table 1. While these various MSCRAMMs are highly similar proteins, they bind to fibrinogen via very heterogeneous binding sites. For example, the C-terminal region of the fibrinogen g-chain is the binding site for ClfA, Fbl, and FnbpA and B (McDevitt et al. 1994; Mitchell et al. 2004; Ni Eidhin et al. 1998). In contrast, ClfB binds to the C-terminal region of the fibrinogen a-chain, and SdrG on the b-chain (Flock et al. 1987; Brennan et al. 2009). Besides *Staphylococci* there are other bacteria, which can engage with fibrinogen. These include the two members of the *Streptococcus* family. *Streptococcus pyogenes* and *Streptococcus mitis* can bind fibrinogen via the M1 protein and lysine, respectively (Cox et al. 2011). While serving as an indirect receptor for bacterial adhesion to platelets, $\alpha_{IIb}\beta_3$ can also be directly bound by several bacterial surface proteins, which is discussed in more detail below.

Involvement of Glycoprotein Ibα (GPIbα)

GP1bα is an essential and integral part of the von Willebrand factor (vWF) binding complex on the platelet surface, which is comprised of GPIbα, GPIbβ, GPIX, and GPV (Bennett et al. 2009). The GPIb/IX/V complex is a key mediator of primary hemostasis. In addition, some bacteria are capable of binding to vWF, docking onto the platelet surface via engagement of the GPIb/IX/V complex. Similarly, protein A from *S. aureus* is capable of binding to vWF, promoting thrombus formation in vitro and under conditions of flow (Claes et al. 2014; O'Seaghdha et al. 2006; Thomer et al. 2013). *Helicobacter pylori* also directly binds to platelet vWF, although the ligand on *H. pylori* mediating this interaction has not yet been identified. These interactions ultimately cause platelet aggregation (Byrne et al. 2003). Besides the vWF-mediated binding of GPIb by bacteria, the glycoprotein can also be directly bound with some bacterial surface proteins, a mechanism discussed in more detail below.

Binding to Platelets via Complement Receptors

It is well known that the complement system can activate platelets by inducing the expression of procoagulant factors, such as prothrombinase complex, on the surface of the cells (Peerschke et al. 2010). Furthermore, both complement pathways—the conventional as well as the alternative pathway—are known to interact with bacteria via complement proteins bound to the bacterial surface (Gadjeva 2014). Upon activation, platelet surface expression of gC1q-R, the receptor for C1q, increases. This enables platelets to indirectly bind bacteria coated with C1q on their surface (Peerschke et al. 2003). Moreover, platelet activation also triggers the increased surface expression of CD62P, a potent

Table 1 Bacterial adhesion to platelets. MSCRAMM and their binding partners

MSCRAMM	Expressing bacteria	Ligand on platelet
ClfA/ClfB	*Staphylococcus aureus*	$\alpha_{IIb}\beta_3$ via fibrinogen/fibronectin
FnbpA/FnbpB	*Staphylococcus aureus*	$\alpha_{IIb}\beta_3$ via fibrinogen
Fbl	*Staphylococcus lugdunensis*	$\alpha_{IIb}\beta_3$ via fibrinogen
SdrG	*Staphylococcus epidermidis*	$\alpha_{IIb}\beta_3$ via fibrinogen Direct $\alpha_{IIb}\beta_3$ interaction
M1	*Streptococcus pyogenes*	$\alpha_{IIb}\beta_3$ via fibrinogen
Lysin	*Sytreptococcus mitis*	$\alpha_{IIb}\beta_3$ via fibrinogen
Isd	*Staphylococcus aureus*	Direct $\alpha_{IIb}\beta_3$ interaction
PadA	*Streptococcus gordonii*	Direct $\alpha_{IIb}\beta_3$ interaction

Examples of microbial surface components recognizing adhesive matrix molecules (MSCRAMM), used by bacteria enabling them to directly or indirectly interact with platelets surface receptors. Clf, clumping factor; Fnbp, fibronectin-binding protein; SdrG, serine–aspartate repeat protein; Isd, iron-regulated surface determinant; Pad, platelet adherence protein

binding partner for the complement protein C3b, leading to platelet-complement factor-coated bacteria interaction (Hamad et al. 2010). Complement-mediated platelet–bacterial interactions were also demonstrated for *S. sanguinis* and *S. aureus*, which induce complement-dependent platelet aggregation (Ford et al. 1996; Loughman et al. 2005).

Complement bacteria–platelet interactions provide a mechanism by which platelets may facilitate the destruction of bacteria by enhancing the complement cascade via surface-bound complement factors and exposed complement receptors. This process may also result in the potential destruction of platelets themselves by being targeted by the lytic activity of the complement system. The complement modulatory potential demonstrated by platelets highlights the immunologic function of this dynamic anucleate cell.

Expression and Functional Significance of the FcγRIIa Receptor

While the expression of the immune receptor FcγRIIa was previously thought to be reserved for classic immune cells, blood platelets also surface-express this receptor (Cassel et al. 1993), although only the Fcγ receptor has been identified in platelets to date. The general function of the immune receptor of the FcγRIIa class is to recognize and bind the Fc domain of immunoglobulin G (IgG). The Fcγ receptor demonstrates a higher affinity for complexed IgG and has a weaker affinity for monomeric IgG. Platelet FcγRIIa is also a key mediator of heparin-induced thrombocytopenia, where autoantibodies recognize the platelet factor 4 (PF4)–heparin complexes that bind to the platelet FcγRIIa (Greinacher 2015). In addition, bacteria recognized and bound by IgG can be attached to this platelet receptor and subsequently internalized (Worth et al. 2006). As platelets are the second most abundant circulating blood cell and express the FcγRIIa in a high copy number on their surface (Cox et al. 2011), platelets may be a significant, yet underappreciated mediator, of antibacterial responses during human infectious settings.

Direct Interactions

Role of Integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa)

In addition to indirect platelet–bacterial interactions utilizing platelet surface receptors traditionally recognizing extracellular matrix, those receptors directly engage with bacterial surface proteins. The aforementioned *S. epidermidis* surface protein SdrG, in addition to binding fibrinogen, also directly targets platelet glycoprotein IIb–IIIa (Brennan et al. 2009). Furthermore, *S. aureus* uses one of its iron-regulated surface determinant (Isd) proteins (IsdB), which are usually used to ensure iron supply for the microbe via heme binding, to bind to integrin $\alpha_{IIb}\beta_3$ in the absence of plasma protein (Miajlovic et al. 2010). Specificity of this interaction was demonstrated using platelets treated with $\alpha_{IIb}\beta_3$ antibodies, a treatment resulting in complete inhibition of platelet–bacterial adhesion. Recently, *S. gordonii* was found to express a surface factor, termed platelet adherence protein A (PadA), which directly interacts with $\alpha_{IIb}\beta_3$ (Petersen et al. 2010).

Interaction with Glycoprotein Ibα (GPIbα)

GPIbα can also serve as a binding ligand by bacteria for direct engagement with human platelets. This interaction involves a family of highly glycosylated, serine-rich bacterial proteins. These proteins include serine-rich protein A (SrpA) from *S. sanguinis* (Plummer et al. 2005), glycosylated streptococcal protein B (GspB), and hemagglutinin salivary antigen (Hsa) from *S. gordonii* (Bensing et al. 2004). Moreover, the staphylococcal accessory regulator (Sar) P protein expressed by *S. aureus* also mediates direct adhesion of bacteria to platelets (Siboo et al. 2005).

Effects of Platelet–Bacteria Interactions on Aggregation

As discussed above, platelet–bacterial interactions include the engagement of platelet surface receptors traditionally viewed as primary mediators of hemostasis. As such, published evidence demonstrates that platelet aggregation is, at least, partially dependent on docking of bacteria with platelets. Once bacteria have bound to platelet surface $\alpha_{IIb}\beta_3$ via fibrinogen or fibronectin, platelet aggregation is initiated in a manner similar as seen with other fibrinogen-coated surfaces (Cox et al. 2011). The SrpA and GspB proteins of *S. sanguinis* and *S. gordonii*, respectively, bind platelets and are requisite for platelet aggregation. If these two proteins are genetically deleted, aggregation is eliminated (Cox et al. 2011). Bacteria-induced platelet aggregation can be shear dependent and shear independent. For example, *S. pyogenes* and *S. aureus* shear forces appear to be dispensable for the induction of platelet aggregation.

There remain aspects of platelet–bacterial interactions and resulting aggregation responses that are incompletely understood. For example, *S. gordonii*, which directly interacts with $\alpha_{IIb}\beta_3$ on platelets through its PadA protein, does not induce platelet aggregation (Petersen et al. 2010). It has been hypothesized that for some of these bacterial groups, binding of GPIb might result in rearrangements that bring other platelet surface receptors, such as FcγRIIa or $\alpha_{IIb}\beta_3$, together in closer proximity, thus promoting thrombus formation (Cox et al. 2011).

In addition to direct interactions leading to aggregation, other mechanisms are involved. Platelet activation induced by surface-bound *S. sanguinis* (via GPIb) results in the release of platelet dense granule content, namely, the vasoactive substances ATP and ADP, which can act via autocrine signaling to further amplify platelet activation. This

mechanism can be enhanced by *S. sanguinis* through the hydrolyzation of ATP to ADP by the surface-expressed ecto-ATPases. The additionally produced ADPs will increase platelet activation using the P2Y signaling pathway (Cox et al. 2011). Several reports highlighted the need for FcγRIIa involvement on platelet aggregation after bacterial adhesion (Cox et al. 2011). Nevertheless, the observed aggregation seemed to be independent of IgG, and therefore FcγRIIa receptors may have alternative functions in addition to what has been traditionally described. In this context, two publications reported a functional interplay between FcγRIIa and GPIb during bacterial stimulation, which might be the first step in signal transduction (Sullam et al. 1998; Sun et al. 1999). Since GPIb binds to FcγRIIa using the same binding motif as when binding to actin, one might speculate that an alternative functional role of FcγRIIa is mediated by cytoskeletal remodeling (Sun et al. 1999). Similarly, Newman et al. showed that the Src residue from $\alpha_{IIb}\beta_3$ phosphorylates the immunoreceptor tyrosine-based activation motif (ITAM) residue of FcγRIIa and thus amplifies the platelet activation signals (Boylan et al. 2008).

In other aspects, bacteria-induced platelet aggregation may be distinct from conventional agonist (ADP, ATP, and thrombin)-induced aggregation. For example, in "binary" aggregation, there is a bacterial density below which platelet aggregation is not observed and above which platelet aggregation is maximal (Kerrigan and Cox 2010). Furthermore, in the presence of bacteria, the lag time before platelet aggregation begins is longer than that observed with hemostatic activation. For some bacteria the lag time might be as long as 20 min, although in some settings, the lag time is dependent on bacterial density (Kerrigan and Cox 2010). There could be several explanations for these lag time variations, including different times required for platelet to bind and interact with bacteria or induction of a weak receptor response.

Bacterial Toxins and Platelets

It is well recognized that bacteria secrete toxins into the host circulation or release endotoxins when lysed or destroyed in other fashion. Members of the *Staphylococcus* family express alpha-toxin, which binds to the lipid bilayer membrane of platelets to form a pore, followed by a flow of calcium, similar to that induced by calcium ionophore (Arvand et al. 1990). Alpha-toxin-induced platelet activation was described to induce RNA splicing and protein synthesis events in platelets as well as the formation of platelet–bacterial aggregates (Rondina et al. 2011; Schubert et al. 2011). Other pore-forming toxins engaging with the platelet surface have been described. These include streptolysin O from *S. pyogenes* (Bryant et al. 2005) and pneumolysin from *Streptococcus pneumoniae* (Johnson et al. 1981). Furthermore, *Porphyromonas gingivalis* secretes a family of cysteine proteases called gingipains. These secreted toxins recognize the platelet protease-activated receptor (PAR) 1, cleaving it in a similar fashion to the natural ligand thrombin, thereby making it functional (Fitzpatrick et al. 2009). Some bacterial toxins have known superantigenic effects. Among those toxins are the family of staphylococcal superantigen-like (SSL) toxins, released by *Staphylococcus aureus* and *S. pyogenes.* One of them, SSL5, can directly interact with GPIba as well as having a direct affinity for GPIV (Cox et al. 2011).

Lipopolysaccharide (LPS) is one of the most important endotoxins expressed on the surface of Gram-negative pathogens. It can be recognized by toll-like receptors (TLRs) expressed by platelets. Platelet surface-expressed TLR-4 is capable of interacting with LPS, leading to platelet activation, P-selectin surface exposure, pre-mRNA splicing events, induction NETs (see later paragraphs), and protein synthetic events and may also contribute to the thrombocytopenia that commonly occurs in sepsis (Akinosoglou and Alexopoulos 2014; Rondina et al. 2011; Semple et al. 2011).

Bacterial-Triggered Release of Platelet-Derived Immunomodulatory Factors

Numerous inflammatory and immune modulating factors are stored in platelet α- and dense granules (Table 2). These molecules are released during platelet activation, modifying host immune responses (Fong et al. 2011; Karshovska et al. 2013; Vieira-de-Abreu et al. 2012; Weyrich and Zimmerman 2004). We will focus in this chapter on several of these molecules and their key signaling events. CD40L (CD154) is a well-established immunoregulatory molecule released by platelets. Platelets were also identified as the major source of circulating soluble CD40L (sCD40L) (Andre et al. 2002). It is known that bacterial ligands interacting with platelet surface TLR-2 (Assinger et al. 2012; Assinger et al. 2011; Cognasse et al. 2007) and TLR-4 (Assinger et al. 2012; Berthet et al. 2012; Cognasse et al. 2007; Ward et al. 2005) induce the release of sCD40L. CD40L is first exposed on the platelet surface membrane in trimeric form (the biologically most active form) and is then cleaved by proteolytic activity, yielding a soluble biologically active fragment. Although not definitely established, there is some evidence suggesting that matrix metalloproteinase (MMP)-9 cleaves the trimeric form of CD40L. The fragment, sCD40L, is recognized by a receptor, CD40, on B lymphocytes, monocytes, and endothelial cells (Blumberg et al. 2009). It should also be pointed out that the soluble form of platelet CD40L may also have an autocrine effect due to the presence of CD40 on the platelet surface (Garraud et al. 2013). sCD40L can facilitate T and B cell interactions leading to antibody production or inducing

Table 2 Inflammatory, antimicrobial, and immunomodulatory factors released by platelets

Function	Factor	Reported target cell
Pleiotropic inflammatory and immune modulators	*Histamine* *5-HT; serotonin*	ECs, monocytes, PMNs, NK cells, T and B cells, eosinophils Monocytes, macrophages
Inflammatory and immunomodulatory lipids	*TXA_2* *PAF*	Platelets, T lymphocyte, and macrophage subsets Platelets, PMNs, monocytes, macrophage, and lymphocyte subsets
Growth factors with immune activities	*PDGF* *TGF-b*	Monocytes, macrophages, T lymphocytes Monocytes, macrophages, T and B lymphocytes
Chemokines	*NAP2 (CXCL7) and related TG variants* *PF4 (CXCL4)* *GRO-a (CXCL1)* *ENA-78 (CXCL5)* *RANTES (CCL5)* *MIP-1a (CCL3)* *MCP-3 (CCL7)*	PMNs PMNs PMNs PMNs Monocytes, eosinophils, basophils, NK cells, T lymphocytes, and DC subsets Monocytes, eosinophils, basophils, NK cells, lymphocytes, and DC subsets Monocytes, basophils, NK cells, lymphocytes, and DC subsets
Antimicrobial peptides	*Thrombocidins* *hBD-1, 2, 3*	No human cellular targets identified; microbicidal for several bacteria and fungi No human cellular targets identified; microbicidal for several bacteria
Cytokines	*IL-1b* *HMGB1* *sCD40L*	Monocytes, ECs, dendritic and macrophage subsets Macrophages, PMNs, ECs Monocytes, B lymphocytes, ECs, platelets

The table is not comprehensive, and additional signaling molecules are found in the platelet secretome; new factors continue to be identified. Some factors are not only secreted but also perform signaling roles while associated with the platelet plasma membrane or on the surfaces of platelet microparticles (e.g., PAF, IL-1β). Many factors also activate target cells in addition to immune effectors, such as fibroblasts and smooth muscle cells. Thrombocidins and hBDs are examples of a larger group of platelet microbicidal proteins (PmPs)
DC, dendritic cell; ECs, endothelial cells; ENA-78, epithelial neutrophil-activating protein-78; GRO-α, growth-regulating oncogene-α; 5-HT, 5-hydroxytryptamine; hBD, human beta defensin; HMGB1, high mobility group box 1; IL-1β, interleukin-1β; MCP-3, monocyte chemotactic protein-3; MIP-1α, macrophage inflammatory protein-1α; NAP2, neutrophil-activating peptide 2; NK, natural killer; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PF4, platelet factor 4; sCD40L, soluble CD40 ligand; TGF-β, transforming growth factor β; TXA_2, thromboxane A_2

class switching of B lymphocytes (Elzey et al. 2005). In addition, sCD40L released from platelet storage pools (granules) induces cytokines, chemokines, and lipid mediators through the activation of CD40+ cells (Blumberg et al. 2009). These cytokines potentiate and modulate neutrophil functions in defense against Gram-positive and Gram-negative bacteria (Miedzobrodzki et al. 2008). Thus, sCD40L is one archetypal molecule that platelets use to orchestrate host defense responses with lymphocytes, endothelial cells, and other host defense effector cells.

Another platelet cytokine being of importance is platelet factor 4 (PF4), a known platelet kinocidin (this term will be elucidated in more detail in the next paragraph). Numerous studies have shown that PF4 plasma levels increase dramatically in the setting of a microbial challenge in vivo. For example, PF4 plasma levels increase during bacterial septicemia (Lorenz and Brauer 1988) and *streptococcal* nephritis (Mezzano et al. 1992). Soluble PF4 can bind to bacteria through its positive charge, thus forming a new recognition site for IgG and effector immune cells (Krauel et al. 2011, 2012). This mechanism is also true for Gram-negative bacteria, since PF4 presents an affinity for bisphosphorylated lipid A of lipopolysaccharide (LPS) bacteria. The newly formed complex is taken up the phagocytic cells. Platelet PF4 might thus facilitate the clearance of certain bacteria. In addition to the lymphocyte modulatory function, an array of bioactive molecules, released from platelets activated by microbial challenge, are chemoattractants for monocytes and neutrophils. These include platelet-derived growth factor (PDGF), platelet-activating factor (PAF), regulated on activation normal T cell expressed and secreted (RANTES) protein, macrophage inflammatory protein (MIP-1 alpha), monocyte chemoattractant protein, and PF4 (Clark-Lewis et al. 1993; Deuel et al. 1981; Tzeng et al. 1985; Semple et al. 2011; Weyrich and Zimmerman 2004).

Platelets Are an Integral Part of the Antimicrobial Host Defense System

The Internalization of Bacteria

In the 1970s, Clawson and colleagues studied the interaction between platelets and bacteria and observed the internalization of *S. aureus* into some platelets (Clawson 1973; Clawson et al. 1975; Clawson and White 1980). These

observations were confirmed by other investigators, especially for *S. aureus* (Youssefian et al. 2002). Youssefian et al. proposed that platelets have the ability to actively internalize or phagocytose microorganisms. This hypothesis was based on finding bacteria inside of vacuoles that were not connected to the open canalicular system (OCS) (Youssefian et al. 2002). Furthermore, it appears that bacterial internalization resembles morphologic evidence of platelet activation. In turn, platelets seem to gain enhanced capacity for bacterial internalization if been activated by bacteria or even conventional agonists (ADP or thrombin), indicating a common mechanism between activation and internalization. Interestingly, immunolabeling revealed that the engulfing vacuoles and the OCS were composed of distinct antigens. When *S. aureus* was engulfed by platelets, membranes expressed CD62P and $\alpha_{IIb}\beta_3$, but not GPIb, similar expression markers seen an activated platelet membrane. Moreover, endocytotic vacuoles containing *S. aureus* were demonstrated to fuse with α-granules, which contain platelet antimicrobicidal proteins. Because of its close proximity to the plasma membrane and the differential surface marker array, these vacuoles may be formed through invagination of the plasma membrane. Internalization of *S. aureus* has also been demonstrated in settings of ADP-induced platelet activation (Li et al. 2008). Under these activating conditions, *P. gingivalis* can also be internalized by platelets (Li et al. 2008). *P. gingivalis*, however, may also be internalized in the absence of activation, an observation not noted with *S. aureus*. While internalization of both bacteria results in them being found in vacuoles independent of the OCS, these findings suggest there may be unique mechanisms regulating the internalization of different bacterial species.

Platelet binding to bacteria using the FcγRII receptor could also initiate the internalization of IgG-bacteria complexes (Worth et al. 2006). In 2011, Antczak et al. demonstrated that platelets are capable of internalizing IgG-coated polystyrene beads (0.5–1.5 μm diameter) in a FcγRII-specific mechanism (Antczak et al. 2011). This endocytotic mechanism was inhibited by the actin-polymerization inhibitor cytochalasin D, demonstrating that actin cytoskeletal dynamics are an integral process of bacteria internalization. While these and other studies support the concept that platelets can internalize or endocytose bacteria, the final fate of these internalized microbes remains uncertain. Some have suggested that the absence of phagolysosomes in platelets precludes their ability to degrade or destroy bacteria (White 2006). Other lines of evidence suppose that the fusion of bacteria with platelet α-granules, which contain antimicrobicidal proteins, could result in pathogen destruction (Youssefian et al. 2002).

Platelets Release Antibacterial Effector Molecules Contributing to Host Defense

In addition to platelets' ability to directly interact with and internalize bacteria, platelets also release antimicrobial proteins and peptides. In 1901, Gengou demonstrated that the bactericidal activity of a previously identified molecule within serum (Fodor 1887), β-lysin, was derived from cells involved in the clotting of blood in a mechanism that was independent of complement (Gengou 1901). This may have been the first study to identify a role for platelets in antimicrobial host defense responses.

In 2010, Yeaman and colleagues proposed calling these antimicrobial molecules platelet microbicidal proteins (PmPs) (Yeaman 2010). These proteins are also known as thrombocidins. PmPs (predominantly PmP1 and PmP2) are released when platelets are activated by thrombin or bacteria. PmPs differ from classically described defensins by their molecular mass, their sequence, and the chains of lysine and arginine residues, which gives them a cationic charge. As with PARs (protease-activated receptors), PmPs must be cleaved by thrombin to become fully functional. The two PmP subunits then act in an autonomous, but complementary, manner, by alternating the permeability of the bacterial wall (Yeaman 2010). The ATP/ADP signaling pathway, utilizing the P2 receptors, primarily triggers the release of PmPs. The initial signal is further amplified by the release of platelet ADP, resulting in potentially autocrine activation of even neighboring platelets (Trier et al. 2008). Over the years, the PmP family was enlarged through the integration of kinocidins, which include those platelet-derived cytokines that have a direct bactericidal effect (Yang et al. 2003). This broad collection of antimicrobial peptides is released from human platelets following thrombin or bacterial stimulation. Similar to chemokines, kinocidins have been organized into classes, which are related to their structural immunobiology. The hallmark CXC motif is the defining element of α-kinocidins. This group includes PF4 (platelet factor 4), platelet basic protein (PBP), connective tissue-activating peptide (CTAP3), and neutrophil-activating peptide (NAP2). In contrast, RANTES is a member of the β-kinocidins, containing the typical CC-chemokine motif. Importantly, members of the α-kinocidin and the β-kinocidin subgroups have synergistic effects. For example, CTAP3 does not have an effect on the viability of *E. coli*. However, the presence of PF4 potentiates CTAP3 activity and thereby reduces the bacterial density significantly. This result is not obtained for PF4 alone (Tang et al. 2002). Using elaborate structural biochemical analyses, the 60–74 structural domain in PF4 was identified as being responsible for this synergistic activity (Yeaman et al. 2007). Kinocidins still also maintain their

primary role, which is the chemoattraction of leukocytes. This mechanism fosters cooperation between platelet and leukocyte factors in bacterial clearance, thus integrating different mechanisms in innate immunity (Agerberth and Gudmundsson 2006; Yeaman et al. 2007).

Kraemer et al. demonstrated that *S. aureus* growth decreases in the presence of human platelets (Kraemer et al. 2011). This platelet bactericidal effect was attributed to the expression of human β-defensin-1 (hBD-1), an antimicrobial peptide primarily expressed by epithelial cells, but also detectable in human blood (Fang et al. 2003). hBD-1 is present in $CD34^+$ hematopoietic progenitor cell-derived megakaryocytes and human platelets at both the messenger RNA (mRNA) and protein level and was localized to an extragranular cytoplasmic compartment. Moreover, treating platelets with *S. aureus*-derived α-toxin, a bacterial pore-forming product, resulted in hBD-1 secretion. Subsequent studies have found that other family members, including hBD-2 and hBD-3, are present in human platelets and possess potent microbicidal activity (Tohidnezhad et al. 2011, 2012).

Boilard and colleagues recently extended these findings, describing a new mechanism by which platelets modulate inflammatory responses by using bactericidal phospholipase (Boudreau et al. 2014). They demonstrate that activated platelets release respiratory-competent mitochondria inside microparticles or as free organelles. The authors further show that the platelet-derived mitochondria are a substrate for the secreted phospholipase A_2 IIA ($sPLA_2$-IIA), a phospholipase otherwise specific for bacteria. This substrate specificity may reflect the ancestral *Proteobacteria* origin of mitochondria. Upon mitochondrial membrane phospholipid hydrolysis by $sPLA_2$-IIA, platelets released a number of lysophospholipids (lyso-PAF, lyso-PC, lysoPE) and free fatty acids. The release of these products resulted in damaged structural integrity and the generation of mitochondrial DNA (mtDNA), a potent pro-inflammatory molecule. Taken together, these and other observations suggest that platelets are involved in infectious immune responses both directly through the release of antimicrobial factors and indirectly through the release of cytokines and other mediators.

Role of Platelets in the Development and Pathophysiology of Bacterial Sepsis

Sepsis is a common and often lethal infectious syndrome that is increasing in incidence. Dysregulated immune and hemostatic responses to pathogens, pathogen-associated molecular patterns, and microbial toxins are central to the pathogenesis of septic syndromes (Fig. 2) (Vincent and Abraham 2006).

Coagulopathy Induced by Septic Triggers

Up to 30–50 % of patients with severe sepsis or septic shock develop disseminated intravascular coagulation (DIC). DIC, in conjunction with peripheral consumption and destruction of platelets, impaired thrombopoiesis, and sequestration of platelets in the microvasculature in platelet–fibrin deposits contributes to thrombocytopenia in sepsis. Moreover, severe

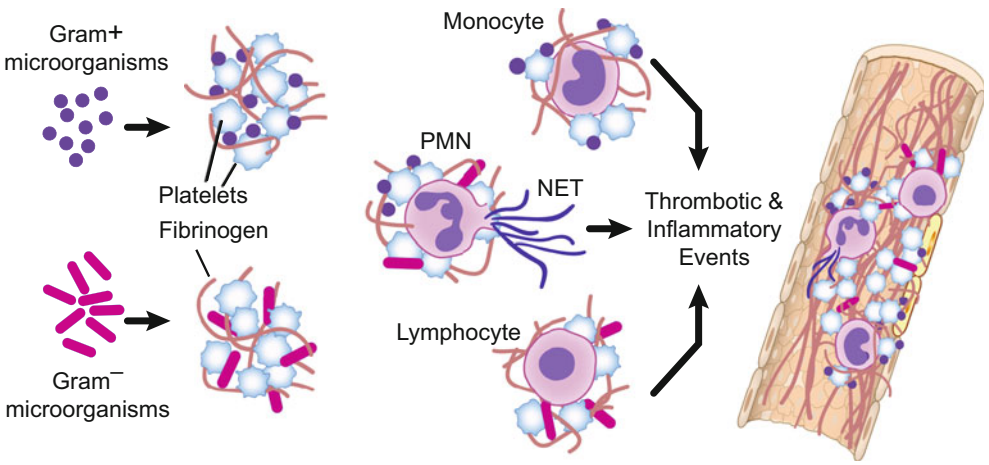

Fig. 2 Platelets interact with bacteria, recruit immune cells, and subsequently trigger inflammatory and thrombotic events. Activation of platelets by bacteria or microbial toxins can trigger platelet–bacterial aggregation or promote the interaction of activated platelets with monocytes, lymphocytes, and neutrophils. Interaction of platelets with leukocytes can also lead to NETosis and bacterial destruction. Platelet or platelet–bacteria/immune cell aggregate sequestration in microvascular beds can mediate increased local inflammatory signaling. In addition, the interaction with fibrinogen and the deposition of fibrin is a central pathophysiological process in sepsis. Furthermore, fibrin degradation products can modify platelet–cell interactions and contribute to the dysregulated hemostasis characteristic of sepsis

thrombocytopenia or a delayed recovery in the platelet count is associated with adverse outcomes from sepsis. As a result of the inflammatory response in sepsis, neutrophils synthesize and release tissue factor, which may initiate or amplify activation of the coagulation cascade, ultimately activating circulating platelets. Activated platelets have increased surface expression of adhesion molecules, promoting interactions with and binding to the damaged endothelial surface and subendothelial matrix proteins (Warkentin et al. 2003). Dysregulated activation of coagulation cascade leads to the deposition of platelet–fibrin thrombi and platelet sequestration in microvessels (Warkentin et al. 2003). Moreover, the formation of homotypic platelet–platelet aggregates and heterotypic platelet–bacteria, platelet–monocyte, and platelet–neutrophil aggregates (in conjunction with excessive thrombin generation and conversion of fibrinogen to fibrin) may further contribute to micro- and macrovascular sequestration and thrombosis (Evans et al. 1969; Gawaz et al. 1995). Thus, platelets are key mediator and effector cells involved in the pathophysiology of sepsis.

The Inflammatory Face of Platelets during Sepsis

First evidence for the inflammatory potential of platelets in sepsis comes from studies demonstrating that the amount of circulating sCD40L is increased in patients with sepsis when compared to healthy control subjects, although levels of sCD40L were independent of sepsis severity (Chew et al. 2010; Inwald et al. 2006; Lorente et al. 2011; Luo et al. 2014; Rahman et al. 2013; Zhang et al. 2011). In murine models of sepsis, sCD40L is released from platelets (Rahman et al. 2009), and matrix metalloproteinase-9, which cleaves platelet-derived CD40L, is increased (Rahman et al. 2012). sCD40L is a key molecule for the recruitment of neutrophils, via macrophage-1 antigen (Mac-1) expression on neutrophils to sites of infection or injury. Mice deficient in CD40L cannot activate neutrophils and do not develop edema or neutrophil infiltration in the lungs in settings of sepsis induced by cecal ligation puncture (CLP) (Asaduzzaman et al. 2009; Rahman et al. 2009).

Another host synthesized pro-inflammatory mediator generated during sepsis is PAF (Yost et al. 2010). PAF binds to its receptor, platelet-activating factor receptor (PAFR), a G-protein coupled receptor expressed by immune cells and platelets, leading to platelet degranulation, the release of inflammatory factors, and initiation of the coagulation cascade. The PAFR-related signaling cascade is usually tightly regulated to prevent excessive thrombo-inflammatory response. Nevertheless, during sepsis, this regulatory balance is shifted, and excessive PAF signaling leads to the activation of neutrophils, monocytes, and platelets, as well as to the formation of heterotypic platelet aggregates (Yost et al. 2010). As discussed above, activation of circulating platelets by bacterial triggers also causes the expression of cell surface receptors and the release of molecules that may further amplify immune responses (Semple et al. 2011). P-selectin (CD62P), which is expressed on the surface of platelets, and its receptor, P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, are additional molecules with key functions during inflammatory and immune responses (Diacovo et al. 1996; Katz et al. 2011; Russwurm et al. 2002; Semple et al. 2011). The functional cross talk between P-selectin and PSGL-1 in conjunction with the leukocyte integrin $\alpha_M\beta_2$ (Mac-1) helps orchestrating the molecular events necessary for leukocyte recruitment (Semple et al. 2011; Weyrich and Zimmerman 2004).

Platelet Microparticles Fuel the Inflammatory Fire in Sepsis

Increased levels of circulating microparticles are also commonly detected in sepsis patients (Ogura et al. 2001). These membrane-enclosed vesicles can arise from different cell types, including granulocytes, monocytes, endothelial cells, and platelets (George 2008). Here, we will focus on platelet-derived microparticles in sepsis. Platelet microparticles (PMPs) are small phospholipid vesicles between 100–1000 nm in diameter that are released after budding from the platelet plasma membrane. As a result, PMP express the same antigens as their parent cells, i.e., $\alpha_{IIb}\beta_3$, GPIb, CD31, CD61, and CD62P (Vasina et al. 2010). Platelet-derived microparticles comprise the majority (e.g., approximately 70–90 %) of the pool of circulating microparticles (Burnier et al. 2009). In sepsis, microparticles can be produced via the stimulation of platelet TLR-4 by bacterial LPS (Brown and McIntyre 2011; Hashimoto et al. 2009; Kappelmayer et al. 2013), but also in response to the Shiga toxin (Ge et al. 2012). Formation of platelet microparticles results in an asymmetrical distribution of membrane phospholipids (Flaumenhaft et al. 2010; Italiano et al. 2010). Therefore, PMPs express highly procoagulant phosphatidylserines (PS) on their surface, although microparticles derived from platelets may be less procoagulant than monocyte-derived microparticles (Owens and Mackman 2011). Furthermore, in recent studies it was shown that two distinct subpopulations of microparticles exist: (1) PS-expressing and (2) PS-nonexpressing MPs. As surface phosphatidylserine-positive microparticles participate in the coagulation process, these data suggest that PS-nonexpressing MPs might have roles separate from the maintenance of primary hemostasis (Arraud et al. 2014; Cloutier et al. 2013).

Platelet microparticles are also capable of delivering immunomodulatory factors, such as IL-1β (Boilard et al. 2010), as well as interacting with other cells. For example, Provost et al. recently demonstrated that human platelet-derived microparticles contained functional Argonaute 2 (Ago2)–miR-223 complexes that were capable of regulating the expression of two endogenous genes in endothelial cells (Laffont et al. 2013). These findings support the concept that microparticles may act as intercellular carriers of functional cell components, thereby influencing the function of other circulating cell types. Boilard et al. even further refined the concept of microparticles being transport vehicles. These investigators demonstrated that platelet-derived microparticles were internalized by activated neutrophils in the endomembrane system via 12(S)-HETE, a hydroxyeicosatetraenoic acid produced through the activity of $sPLA_2$-IIA, which is present in inflammatory fluids (Duchez et al. 2015). Platelet microparticles can also be internalized by macrophages, leading to the differential expression of numerous miRNAs and mRNAs, including transcripts encoding for chemokine (C–C motif) ligand 4 (CCL4/MIP-1β), colony-stimulating factor 1 (CSF1), and TNF (Laffont et al. 2015). Additionally, platelet microparticle uptake by macrophages led to an increase in their phagocytic capacity (Laffont et al. 2015). These recently discovered functions of platelets and platelet-derived microparticles highlight once more the versatility of this anucleate cell and its key roles in inflammatory and immune disorders.

Platelets Induce NETosis in Sepsis

NET formation in response to bacterial invasion was first demonstrated by Brinkmann and colleagues in 2004 (Brinkmann et al. 2004). NETs consist of expulsed nuclear DNA that forms lattices, containing histones (H1, H2A, H2B, and H4), granule enzymes (elastase, myeloperoxidase), and antimicrobial proteins released by the PMN in parallel with extrusion of nuclear material (Brinkmann et al. 2004). NETs can be generated under both flow and static conditions, although there is some evidence that flow conditions enhance bacterial trapping (analogous to the human circulatory system) (Clark et al. 2007). NETs are long lattices that can be as large as 25 nm in diameter (Clark et al. 2007). These DNA/enzyme complexes accomplish both capture and killing of extracellular bacteria. In addition, antimicrobial activities can be blunted or subverted by endonucleases and DNases expressed by some microorganisms (Beiter et al. 2006; Buchanan et al. 2006). NETs are also implicated in vascular injury associated with neutrophil–platelet interactions in human sepsis (Clark et al. 2007). For example, Ma and Kubes demonstrated that a bacterial environment or stimulation using LPS resulted in the in vivo formation of NETs which trapped bacteria, reducing their growth, and blunted the severity of sepsis (Ma and Kubes 2008). Ma and colleagues further demonstrated that NETs were able to trap both Gram-negative and Gram-positive bacteria.

NETosis is an active mechanism occurring within 5–10 min after neutrophil stimulation and being distinct from apoptosis and necrosis (Brinkmann et al. 2004). There is evidence that reactive oxygen species (ROS) generation is a key event in NET formation (Fuchs et al. 2007; Yost et al. 2009). However, ROS generation and NET formation can be dissociated under some conditions, suggesting that the pathways and mechanisms regulating NET formation are more complex than previously appreciated (Chow et al. 2010; Marcos et al. 2010). Yost et al. found that the mammalian target of rapamycin (mTOR) regulates NET formation through induction of hypoxia-inducible factor 1α (HIF-1α). Citrullination of histones, release of mtDNA, and platelet hBD-1 also mediate key aspects of NET formation (Kraemer et al. 2011; Yousefi et al. 2009). In addition, platelet-derived microparticles containing mitochondria as well as mitochondria released from platelets into the circulation have been linked to NET formation. The hydrolysis of the mitochondrial membrane by $sPLA_2$-IIA also leads to the formation of inflammatory mediators inducing robust NET formation in an in vitro assay (Boudreau et al. 2014).

Nevertheless, while NETs may be key host defense mechanisms in response to bacterial invasion, NET formation may also have adverse effects on the microvascular circulation by promoting the formation of microthrombi, thereby also potentially preventing immune cells from reaching the bacteria. Emerging evidence implicates NET formation as injurious in many inflammatory and thrombotic diseases, including sepsis (Xu et al. 2009), coronary artery disease (Borissoff et al. 2013), microvascular thrombosis (Xu et al. 2009), and deep vein thrombosis (Fuchs et al. 2010). Recent work by Wagner and colleagues demonstrates that thrombin-stimulated platelets induce NET formation via P-selectin/PSGL-1 interactions (Etulain et al. 2015). These intriguing findings suggest that the P-selectin/PSGL-1 axis may be a potential therapeutic target for settings where dysregulated NET formation is considered injurious (Etulain et al. 2015).

Conclusion

Established and emerging evidence supports the role of platelets as dynamic sentinels and effector cells during bacterial infections. These previously unrecognized functions broaden the view of platelets as having complementary roles in both hemostasis and immune pathways. Furthermore, as platelets orchestrate key aspects of immune and

inflammatory responses, platelets are being studied as a potential therapeutic target in sepsis.

Take Home Messages

- Platelets are key sentinels and effector cells involved in the pathophysiology of sepsis.
- Platelet integrins mediate platelet–bacterial interactions.
- FcgRIIa on platelets coordinates antibacterial host responses.
- The final fate of internalized bacteria remains unclear and warrants further investigations.
- Platelets are involved in infectious immune responses both directly through the release of antimicrobial factors and indirectly through the release of cytokines and other mediators.
- The P-selectin/PSGL-1 axis may be a potential therapeutic target for settings of injurious NET formation.

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Virus–Platelet Associations

Edward L.G. Pryzdial, Bryan H. Lin, and Michael R. Sutherland

Abstract

Virus–platelet interplay is complex. Diverse virus types have been shown to associate with numerous distinct platelet receptors. This association can benefit the virus or the host, and thus the platelet is somewhat of a renegade. Evidence is accumulating to suggest that viruses are capable of entering platelets. For at least one type of RNA virus (dengue virus), the platelet has the necessary post-translational and packaging machinery required for production of replicative viral progeny. As a facilitator of immunity, the platelet also participates in eradicating the virus by direct and indirect mechanisms involving presentation of the pathogen to the innate and adaptive immune systems, thus enhancing inflammation by release of cytokines and other agonists. Virus-induced thrombocytopenia is caused by tangential imbalance of thrombopoeisis, autoimmunity, and loss of platelet function and integrity.

Introduction

The historical view that platelets solely participate as primary facilitators in hemostasis and thrombosis is now outdated. Reminiscent of roles played by their evolutionary counterparts in primitive organisms (Delvaeye and Conway 2009), it is now well-established that platelets directly modulate cells of the immune system through receptor-mediated cell contact with leukocytes and pathogens. In combination with these interactions, external stimuli induce secretion of cytokines and other cell modulators from platelet α-granules and dense granules to produce additional indirect effects on the cellular immune response. Moreover, in contrast to the bygone paradigms, the platelet proteome is not static. Platelets inherit from antecedent megakaryocytes a repertoire of mRNAs and the necessary post-transcriptional machinery to alter their protein composition (Schubert et al. 2014). Platelets survey their local environment, reacting dynamically to change, and are thus pivotally positioned during the defense against pathogens (Semple et al. 2011). Here, we focus on their complex interactions with viruses.

Many excellent review articles are available that recognize the importance of platelets in virology. In some cases, these highlight the effects of specific virus types (Chabert et al. 2015; Hottz et al. 2011), whereas others are more general (Assinger et al. 2014; Zapata et al. 2014). The ultimate message is that viruses trigger a complexity of biochemical and cellular events, often resulting in diminished platelet count, altered vascular permeability, and consequent bleeding diathesis. In this review, we revisit these ideas and add a temporal aspect to the emerging model. We speculate that platelets are more than immune modulators and "innocent" bystanders in the host response to viral infection and that, in the case of viruses with an RNA genome, platelets initially participate as a viral ally.

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Virus Binding to Platelets

Platelets are not considered to be a primary host cellular target in virology. Therefore, to identify the receptors that bind viruses, other cell types are usually investigated. The virus–cell interaction is the key initiating step in the virus lifecycle and a logical target for development of antiviral agents. Based on the knowledge of receptors presented on typically studied cells, predictions can be made about whether platelets express the necessary receptors to facilitate specific virus interactions. To help explain platelet-related pathology as a result of infection of certain viruses, direct binding has been reported. Here we loosely divide these interactions on the basis of two principal hemostatic diseases in which direct virus–platelet interactions could logically be involved: thrombocytopenia and hemorrhagic fever (HF).

Thrombocytopenic Viruses When a foreign particle, such as a virus, binds directly to the platelet surface, it is reasonable to speculate that consequent immune recognition leads to a reduction in platelet count. Thus, virus-induced thrombocytopenia has been the rationale for investigating interactions between viruses and platelets. Table 1 lists viruses that correlate with thrombocytopenia without HF. Representing at least six virus families, these viruses range broadly in structure and genome organization and, with the exception of adenoviruses, are surrounded by a lipid bilayer envelope that contains both host cell- and virus-derived elements. Therefore, an adaptive immune response as a result of viral infection of platelets may involve both virus antigens and co-epitopes originating from virus and host factors, resulting in a reduced platelet count. Receptors on the platelet that associate with viruses have been identified and include integrins α5β1, $\alpha_{IIb}\beta_3$, and α5β3; the lectin, dendritic cell-specific intercellular adhesion molecule-3-grapping non-integrin (DC-SIGN); Toll-like receptors (TLRs) 2 and 4; coxsackie-adenovirus receptor (CAR); complement receptor 2 (CR2); C-X-C chemokine receptor type 4 (CXCR4); C-type lectin domain family 2 (CLEC-2); chemokine C-C motif ligand (CCL); and glycoprotein (GP) VI. Co-receptor systems involving more than one virus–platelet interaction may also exist, as in human immunodeficiency virus (HIV). In some cases, the platelet receptor is unknown (e.g., SARS-CoV).

A variety of methods have been used to establish specific receptors. Early inhibition studies involving adenovirus used purified matrix proteins, adenovirus penton base proteins, and synthetic peptides. Results suggested the importance of the α5 and β1/β3 integrins for infection of several cell lines (Stevenson et al. 1997; Wickham et al. 1993). Immuno- and electron microscopy studies have shown that platelet $\alpha_{IIb}\beta_3$ is also important for the binding of adenovirus (Gupalo et al. 2013). Further cellular studies implicated CAR (Bergelson et al. 1997) in the virus–platelet association and identified this receptor on platelets by flow cytometry and RNA isolation (Othman et al. 2007). Additional studies using immuno-inhibition and arginyl–glycyl–aspartyl (RGD) motif peptide mimics confirmed the importance of α5 integrins in adenovirus binding to platelets; however, they failed to detect CAR expression (Shimony et al. 2009).

Hepatitis C virus (HCV) is regularly associated with thrombocytopenia (Weksler 2007). Platelet glycoprotein GPVI has been implicated in HCV–platelet interaction through peptide and immuno-inhibition studies (Pugliese et al. 2004), purified protein assays, and virus binding assays (Zahn et al. 2006) and was shown to be important for infection and dissemination (Ariede et al. 2015; Zahn et al. 2006). DC-SIGN has also been demonstrated to be involved in HCV binding.

As in infection of other cell types, HIV has been shown to associate with platelets through a variety of cell-surface receptors (Youssefian et al. 2002). Platelet DC-SIGN, as identified by flow cytometry, western blotting, and PCR, can recognize and bind pathogen-associated molecular patterns (PAMPs) on HIV because immuno-inhibition results in decreased binding (Boukour et al. 2006; Chaipan et al. 2006). Using similar immuno-inhibition and flow cytometric approaches, CLEC-2 was identified as a platelet receptor for HIV (Chaipan et al. 2006). Additional putative receptors for HIV on platelets also include CXCR4, CCL3, and CCL5 (Flaujac et al. 2010).

Platelet integrins appear to serve as the main binding partner because they contain the common RGD motif. Therefore, the presence of integrin-binding sequences in several virus families suggests that integrins are important for platelet association and signaling, with effects leading to thrombocytopenia. Viral envelope glycoproteins can serve as sources of PAMPs and facilitate virus–platelet interaction via TLRs. This is suggested as a mechanism for cytomegalovirus (CMV)-induced thrombocytopenia. Evidence implicating direct binding and consequent cell stimulation as a result of CMV-encoded glycoprotein B and glycoprotein H interactions with TLR2 (Boehme et al. 2006) on platelets or neutrophils has been obtained from immunoprecipitation and immuno-inhibition studies of co-transfected human embryonic kidney and normal fibroblast cells, respectively (Assinger et al. 2014).

Platelets also have on their surface the complement receptor type 2 (CR2), which functions as a receptor for Epstein–Barr virus (EBV), as shown by cell and immuno-inhibition techniques (Ahmad and Menezes 1997; Hutt-Fletcher 2007). Whether this also protects the virus from complement-mediated innate immune clearance is not known.

Platelets have one class of receptor for the Fc domain of antibodies, FcγRIIA. Once virus-directed antibodies are generated by the adaptive immune response, "bridged" interactions can be facilitated by platelet FcγRIIA, as demonstrated for influenza A virus (IAV) (Boilard et al. 2014).

Table 1 Viruses correlating with thrombocytopenia

Virus	TCP	HF	Family	Genome	Receptor on Platelets	Virus Receptor	Other Cell Receptor	Platelet Entry
Adenovirus (Othman et al. 2007, Shimony et al. 2009, Zhang and Bergelson 2005)	Yes	No	Adenoviridae	DNA, non-enveloped	CAR $\alpha_{IIb}ß_3$ $\alpha_5ß_3$ $\alpha_5ß_1$ (?) sialic acid (?) TLR4 (?)	Fiber knob RGD of penton base	$\alpha_5ß_1$, $\alpha_5ß_3$, $\alpha_5ß_5$, TLR3, TLR4, TLR9, sialic acid	Yes
CMV (Agbanyo and Wasi 1994, Assinger et al. 2014, Boehme et al. 2006)	Yes	No	Herpesviridae	DNA, enveloped	TLR2 $\alpha_5ß_1$	gB, gH	TLR DC-SIGN	ND
EBV (Ahmad & Menezes 1997, Hutt-Fletcher 2007)	Yes	No	Herpesviridae	DNA, enveloped	CR2	gp350/220	CR2	ND
VZV (Rand & Wright 1998, Sloutskin et al. 2014, Zhu et al. 1995)	Yes	No	Herpesviridae	DNA, enveloped	HSP (?)	gE	HSP	ND
HSV (Caviness et al. 2008, Spear 2004)	Yes	No	Herpesviridae	DNA, enveloped	HSP (?)	gC, gD	HSP	ND
Influenza (Boilard et al. 2014, Danon et al. 1959, Le et al. 2015)	Yes	No	Orthomyxoviridae	RNA, enveloped	$\alpha_{IIb}ß_3$, FcγRIIa, sialic acid (?)	HA	Sialic acid FcγRIIa	Yes
HIV (Chaipan et al. 2006, Youssefian et al. 2002, Zapata et al. 2014)	Yes	No	Lentivirus	RNA, enveloped	CCL1,3,4 CXCR4 DC-SIGN CLEC-2	ENV protein	DC-SIGN	Yes
HCV (Ariede et al. 2015, de Almeidal et al. 2007, Zahn et al. 2006, Zahn and Allain 2005)	Yes	No	Flaviviridae	RNA, enveloped	DC-SIGN GPVI	E2	DC-SIGN Heparin CD81	ND
SARS-CoV (Imai et al. 2005, Li et al. 2003)	Yes	No	Coronaviridae	RNA, enveloped	CD13	gS	ACE2	ND

Viruses that induce only thrombocytopenia (*TCP*) and not hemorrhagic fever (*HF*) are listed. Those viruses that have an RNA genome (*red*) and are permissible to entry may be replicated by platelets. Viruses encoded by a DNA genome (*green*) cannot be replicated by platelets
αNβN member of the integrin family, *ACE2* angiotensin converting enzyme 2 receptor, *CAR* coxsackie-adenovirus receptor, *CCL* chemokine (C-C motif) ligand, *CD* cluster of differentiation, *CLEC-2* C-type lectin domain family 2, *CMV* cytomegalovirus, *CR* complement receptor, *CXCR4* C-X-C chemokine receptor type 4, *EBV* Epstein-Barr virus, *E* or *ENV* envelope, *DC-SIGN* dendritic cell-specific intercellular adhesion molecule-3-grapping non-integrin, *FcγRII* Fc gamma receptor II, *gp* or *g* glycoprotein, *GPVI* platelet glycoprotein VI, *HA* hemagglutin, *HCV* hepatitis virus C, *HIV* human immune deficient virus, *HSP* heparan sulfate proteoglycan, *HSV* herpes simplex virus, *ND* data not available in the literature, *RGD* arginine–glycine–aspartic acid peptide sequence, *SARS* severe acute respiratory syndrome, *TLR* Toll-like receptor, *VZV* varicella zoster virus, *?* proposed platelet receptor based on indirect cell studies

Making vaccine development difficult, some secondary viral infection mechanisms exploit antibody-dependent enhancement; examples are dengue virus (DENV) and HIV (Guzman et al. 2013). Although antibody-bridged binding to platelets has not been specifically documented for many viruses, it is reasonable to speculate that such viral immune complexes commonly form interactions with platelets. Similar to engagement of other receptors on the platelet surface, these multivalent adducts could crosslink the FcγRIIA, causing platelet activation.

Hemorrhagic Fever Viruses Whereas the bleeding phenotype inherent to thrombocytopenia is considered to be

predominantly caused by loss of platelets, the effects on hemostasis leading to virus-induced HF are typically far more complicated (Zapata et al. 2014). HF is characterized by the loss of blood homeostasis, leading to increased vascular permeability and bleeding, which can progress to shock. The causative agents of viral HF are enveloped RNA viruses from four families: Flaviviridae, Bunyaviridae, Arenaviridae, and Filoviridae (Table 2). These families contain well-known species such as Ebola and DENV that are featured as "headline news" because of the devastating and graphic illnesses these epidemic pathogens can cause. Some of the viruses are especially difficult to experimentally manipulate because of their biohazard classification level and, consequently, relatively little is known about their biochemistry. Nevertheless, receptors for viruses known to cause viral HF have been identified. Interestingly, these are similar to those receptors characterized for viruses as predominantly thrombocytopenic: β1/β3 integrins, lectins, and TLRs. Therefore, it is not surprising that HF viruses can also result in thrombocytopenia.

Using specific antibody inhibition and virological plaque-forming assays, DC-SIGN and heparan sulfate proteoglycan (HSP) were demonstrated to be important in DENV binding to platelets (Hottz et al. 2013b; Simon et al. 2015) Interestingly, additional binding sites were expressed by pretreating platelets with the agonist thrombin (Simon et al. 2015). Whether these are the same type of receptor is unknown. Employing a transduced cell model of infection, DC-SIGN has also been shown to be involved as a receptor in Ebola virus infection (Alvarez et al. 2002). Mutagenesis, binding, and RNA interference experiments have implicated Axl (Shimojima et al. 2007) and Tyro3 (Hunt et al. 2011) in Ebola's complex cell engagement mechanism. These observations suggest that multiple receptors are occupied in the Ebola–platelet interaction. Interestingly, similar platelet surface molecules may bind Lassa virus, as indicated by the identification of DC-SIGN, Axl, and Tyro3 as receptors using cell binding and infection assays (Shimojima et al. 2012). In addition, the results of immunoblot and competition assays involving purified virus suggested that α-dystroglycan is a Lassa virus receptor (Cao et al. 1998). Neutralizing antibody experiments have implicated β3 integrins in the interaction between cells infected with hantavirus and platelets (Gavrilovskaya et al. 1999; Gavrilovskaya et al. 2010). Although cell and platelet receptors remain elusive for some HF viruses, it is possible that a particular receptor may function across an individual virus family.

Virus–Platelet Interactions with Moderate Pathology Although virus–platelet interactions leading to life-threatening effects are well-known, there are also a number of viruses that bind to platelets without serious hematological pathology. Predominately from the Picornaviridae family, these non-enveloped RNA viruses cause enteric, throat, and nasal infections. Although not directly confirmed as analogous functional receptors for picornavirus on platelets, β1 and β3 integrins are known to facilitate interactions with receptors on other cell types. Studies following surface expression of mutated proteins in CHO cells and antibody inhibition studies have identified integrin $\alpha_2\beta_1$ as a binding partner for both rotavirus and echovirus (Coulson et al. 1997; Fleming et al. 2011). Utilizing an antigenic inhibition model and CHO transfection, members of the β1 and β3 integrin families were shown to mediate parechovirus infection (Triantafilou et al. 2000). This suggests the potential for binding recognition with the integrin known to be expressed on the platelet surface. It is somewhat puzzling that thrombocytopenia is not induced because engagement of these receptors by other viruses is consistent with this outcome. Consequently, integrins may not be the only common denominator resulting in this virus-mediated hemostatic disorder.

Virus-Induced Platelet Activation Platelets circulate in a resting state and are stimulated by ligand–receptor engagement. It is clear that protein and carbohydrate receptors on the surface of platelets bind viruses from many distinct families (Tables 1, 2, and 3). Indeed, it is not surprising that the interactions known to exist between viruses and platelet receptors can facilitate platelet activation. Interactions with DENV grown in culture have been shown to induce platelet shape changes, as monitored by atomic force and electron microscopy (Ghosh et al. 2008). DENV has also been demonstrated to cause the exposure of P-selectin and procoagulant phospholipid on the platelet surface (Hottz et al. 2013b; Simon et al. 2015), and to initiate apoptosis-like markers, including caspase activation and mitochondrial permeability changes (Hottz et al. 2013b), Similarly, HIV can induce microparticle release and other hallmarks of platelet activation (Wang et al. 2011) that are related to plasma virus levels (Mayne et al. 2012). Differential response to platelet agonists in HIV-infected individuals points to functional changes in platelets, with implications for thrombosis (Satchell et al. 2010). Interestingly, additional studies further implicated HIV treatment strategies in the development of hyper-responsive platelets (Falcinelli et al. 2013; Gresele et al. 2012). Although the direct effect of virus binding on platelet activation has not been studied extensively, outside-in signal-induced platelet changes are probably a general characteristic, regardless of the type of virus.

Virus Replication by Platelets

Entry Cell binding is the first step used by all viruses to exploit the host's cellular replication apparatus. Without

Table 2 Hemorrhagic fever viruses with putative platelets interactions

Virus	TCP	HF	Family	Genome	Receptor on Platelet	Virus Receptor	Other Cell Receptor	Platelet Entry
Dengue virus (Noisakran et al. 2009, Simon et al. 2015, Tassaneetrithep et al. 2003)	Yes	Yes	Flaviviridae	RNA, enveloped	$\alpha_5ß_3$ DC-SIGN HSP FcγRIIa,	GPE	DC-SIGN, LSIGN	Yes
Yellow Fever	ND	Yes	Flaviviridae	RNA, enveloped				ND
Kyasanur forest disease virus)	ND	Yes	Flaviviridae	RNA, enveloped				ND
Alkhumra virus	ND	Yes	Flaviviridae	RNA, enveloped				ND
Hantavirus (Gavrilovskaya et al. 1999, Gavrilovskaya et al. 2010)	Yes	Yes	Bunyaviridae	RNA, enveloped	$\alpha_{IIb}ß_3$, $\alpha_5ß_1$ (?)	GN, GC	$\alpha_{IIb}ß_3$, $\alpha_5ß_3$, $\alpha_5ß_1$	ND
Rift valley fever (de Boer et al. 2012)	ND	Yes	Bunyaviridae	RNA, enveloped	HSP (?)		HSP	ND
Severe fever with thrombocytopenia syndrome (Jin et al. 2012)	Yes	Yes	Bunyaviridae	RNA, enveloped				ND
Crimean–Congo hemorrhagic fever	Yes	Yes	Bunyavirus	RNA, enveloped				ND
Chikungunia virus (Long et al. 2013)	ND	Yes	Togaviridae	RNA, enveloped		GP	DCIR	ND
Lassa (Kunz et al. 2005, Shimojima et al. 2012, Zapata et al. 2014)	ND	Yes	Arenaviridae	RNA, enveloped	DC-SIGN	GPC	α - DG Tyro3, DC-SIGN, Axl,	ND
Lujo	ND	Yes	Arenaviridae	RNA, enveloped				ND
Machupo	ND	Yes	Arenaviridae	RNA, enveloped				ND
Guanarito	ND	Yes	Arenaviridae	RNA, enveloped				ND
Junin	Yes	Yes	Arenaviridae	RNA, enveloped				ND
Ebola (Alvarez et al. 2002, Shimojima et al. 2007, Zapata et al. 2014)	Yes	Yes	Filoviridae	RNA, non-enveloped	DC-SIGN (?) α5ß1 (?)	GP	Tyro3, DC-SIGN, Axl, $\alpha_5ß_1$, NPC1	ND
Marburg	ND	Yes	Filoviridae	RNA, non-enveloped				ND

Viruses that cause hemorrhagic fever (*HF*) are listed. All known HF viruses are RNA viruses and therefore if entry into platelets is permissible, they can be replicated. Dengue virus is the only one for which evidence of platelet-mediated replication has been investigated. HF viruses that interact with platelets span many virus families, as highlighted by the different colors. Some of these viruses are also known to induce thrombocytopenia (*TCP*) and, although data is not available in the literature (*ND*) for each, it cannot be excluded

αNβN member of the integrin family, *α-DG* alpha-dystroglycan, *Axl* Axl receptor tyrosine kinase, *DC-SIGN* dendritic cell-specific intercellular adhesion molecule-3-grapping non-integrin, *DCIR* dendritic cell immunoreceptor, *G* or *GP* glycoprotein, *HSP* heparan sulfate proteoglycan, *LSIGN* liver/lymph node-specific intercellular adhesion molecule-3-grapping non-integrin, *NPC1* cholesterol transporter Niemann-Pick 1, *Tyro* tyrosine-protein kinase receptor, *?* proposed platelet receptor based on indirect cell studies

Table 3 Virus–platelet interactions with moderate hemostatic effects

Virus	TCP	HF	Family	Genome	Receptor on Platelet	Virus Receptor	Other Cell Receptor	Platelet Entry
Rotavirus (Coulson et al. 1997, Fleming et al. 2011)	No	No	Reoviridae	RNA, non-enveloped	α2ß1 sialic acid (?)	VP4	α2ß1, sialic acid	ND
Coxsackie (Bergelson et al. 1997)	No	No	Picornaviridae	RNA, non-enveloped	CAR	VP1	CAR α5ß3, α5ß5	ND
encephalomyocarditis virus (Koupenova et al. 2014)	No	No	Picornaviridae	RNA, non-enveloped	TLR7			Yes
Echovirus (Triantafilou et al. 2000, Triantafilou and Triantafilou 2001)	No	No	Picornaviridae	RNA, non-enveloped	α2ß1 (?)		α2ß1 α5ß3	ND
Parechovirus (Triantafilou et al. 2000, Triantafilou & Triantafilou 2001)	No	No	Picornaviridae	RNA, non-enveloped	α5ß1 (?)	VP1	α5ß3, α5ß1	ND

Mostly from the Picornavirus family, certain viruses have been reported to interact with platelets without inducing thrombocytopenia (*TCP*) or hemorrhagic fever (*HF*). These interactions may be indicative of the role of platelets in the innate immune response
αNβN member of the integrin family, *CAR* coxsackie-adenovirus receptor, *ND* data not available in the literature, *TLR* Toll-like receptor, *VP* virus protein, *?* proposed platelet receptor based on indirect cell studies

question, platelets have that binding capacity. The second step in the virus lifecycle is cell entry. Two general mechanisms of cell entry can be initiated upon contact between an animal virus and a target host cell. Most viruses traverse the plasma membrane using the endocytic machinery intrinsic to the cell, whereby the virus is engulfed by a vesicular structure for intracellular transport (Ghigo 2010). Within these endosomal compartments, low pH typically induces the release of genetic contents from the viral nucleocapsid. Based on microscopic morphology and biochemistry, nearly a dozen viral endocytosis mechanisms have been discerned (Cossart and Helenius 2014; Mercer et al. 2010). A second entry mechanism is used exclusively by enveloped viruses. After receptor-mediated cell surface binding, both virus- and host-encoded (Derry et al. 2007) proteins on the virus envelope can engage to form a conformational fusion complex that merges the envelope and plasma membrane.

In contrast to the known interactions between viruses and platelets, little evidence of platelet entry has been documented. Immunogold electron microscopy revealed that purified HIV is trapped within platelet endosomal structures (Youssefian et al. 2002). Another entry mechanism was suggested by colocalization of purified adenovirus with the surface-connected channels of the platelet open canalicular system (Stone et al. 2007). Purified IAV and platelets enabled observation by electron microscopy of the surface association and uptake of virus-like particles into vacuolar structures (Danon et al. 1959). Furthermore, virus-like particles have been reported in the platelets of DENV-infected patients, although these difficult experiments lacked confirmation of virus (Noisakran et al. 2009). Electron microscopy has also been used to demonstrate encephalomyocarditis virus (EMCV) uptake by platelets in a murine model of infection (Koupenova et al. 2014). To confirm their endocytic capabilities, purified platelets have been shown to engulf *Staphylococcus aureus* (Youssefian et al. 2002) and liposomes that were engineered for drug delivery (Chan et al. 2015). Although viral entry mechanisms are not known, molecular details following the transfer of synthetic particles into platelets suggests that several simultaneous endocytotic pathways are involved (Chan et al. 2015). These pathways may have dynamin dependence as a common aspect and involve caveolae- or clathrin-mediated uptake (Mercer et al. 2010). Thus, observations of virus uptake by platelets are supported by the availability of entry mechanisms for other particle types.

Viral Protein Synthesis Platelets associate with both DNA- and RNA-containing viruses. Assuming successful entry and release of genetic material into the platelet, only RNA viruses replicate because platelets are DNA transcription incompetent. The list of RNA viruses with known or suspected platelet interactions is extensive and highlighted in Tables 1, 2, and 3. Platelets contain all of the post-transcriptional apparatus necessary for potential assembly

of an infectious RNA virus. To demonstrate that platelets can translate viral RNA, purified platelets inoculated with purified DENV have been shown to produce viral nonstructural protein 1 (NS1), as detected by Western blot analysis (Simon et al. 2015). Because the DENV single-stranded RNA (ssRNA) must first be translated as a polyprotein, the finding that NS1 had the predicted molecular weight of the mature protein implied that the functional DENV protease complex (NS2B/NS3) was also properly translated and processed by the platelets (Simon et al. 2015). Thus, platelets not only facilitate the initial step in the virus lifecycle, specific surface engagement (Tables 1, 2, and 3), but also have the means to allow penetration of the virus into the cytoplasm and occupation of the platelet translational mechanism.

Virus Replication Replication of viral genomic material has been followed as a surrogate for functional virus-encoded polymerase generation. In these studies, the mRNAs of all four serotypes of purified DENV in combination with purified platelets were enhanced (Simon et al. 2015), which substantiated an earlier preliminary report (Onlamoon et al. 2010). These studies were extended using virus plaque formation assays in combination with a translation inhibitor and demonstrated that the virus is properly assembled, resulting in production of infectious progeny by platelets (Simon et al. 2015). Interestingly, platelet units stored under blood bank operating conditions also produced new DENV (Sutherland et al. 2016). A generalized model is presented in Fig. 1, highlighting the emerging concept that platelets could be a reservoir for permissive RNA viruses. The concept is based on (1) significant literature demonstrating that many types of virus bind directly to platelets; (2) several studies showing platelet entry; and (3) generation of infectious DENV progeny by platelets.

Although evidence suggests that platelets could be recruited by RNA viruses as conspirators for replication, it is conceivable that only platelet subsets can fulfill this role. As an example, ~20 % of platelets are positive for DC-SIGN (Hottz et al. 2013b) and may account for binding to HIV, DENV, HCV, Ebola, and LASV, which are known to use DC-SIGN for cell surface attachment (Tables 1, 2, and 3). Furthermore, TLR2 is found on a subset of ~14 % of platelets and is involved in at least the CMV interaction (Boehme et al. 2006). Some platelet-interacting viruses are known to use co-receptor systems, of which candidate receptors are found on platelets. But, whether these too are distributed as subpopulations, like DC-SIGN and TLR2, has not been evaluated. Of these functionally distinct classes of platelets, only some may be permissive to entry and replication after binding.

Platelet Immune Response Against Viruses

Platelets may initially harbor and replicate certain viruses (Simon et al. 2015). But, as known facilitators and modulators of the immune response to pathogen invasion (Jenne and Kubes 2015), platelets also competently present bound viruses to leukocytes for clearance. To enhance this innate immune recognition, the association of viruses with platelets induces the release of cytokines, which causes local infiltration of immune cells. Many platelet surface molecules mediate leukocyte cross-talk and have an essential effect on viral infection. The modulation of platelet function by viruses, leading to their ultimate involvement in innate and adaptive immunity, is summarized in Fig. 2.

Toll-Like Receptors TLRs (Cognasse et al. 2015) are a family of innate immune regulators that recognize pathogen-associated molecular patterns (PAMPs), which are markers associated with viruses, bacteria, and fungi that lead to neutrophil-mediated pathogen destruction. Known to recognize ssRNA, typical of many platelet-interacting viruses (Tables 1, 2, and 3), platelet TLR7 was identified as vital for EMCV clearance by platelets (Koupenova et al. 2014). Interestingly, this receptor is expressed within endosomes and its functional involvement implies endocytosis of the virus by the platelet. Penetrance of EMCV was TLR7 dependent, with activation of TLR7 resulting in the release of the α-granules that house proinflammatory cytokines, leading to neutrophil aggregation, endothelial cell adhesion, and inflammation. The TLR7 platelet response was shown to contribute to host survival, as EMCV levels decreased and there were no observable prothrombotic events as a consequence of potential platelet activation (Koupenova et al. 2014).

Unlike TLR7, TLR2 is expressed on the platelet surface. CMV was found to associate predominately with the TLR2-positive platelet subpopulation (Boehme et al. 2006). As seen using flow cytometry, CMV induces rapid surface expression of P-selectin, leading to the release of proinflammatory CD40 ligand and interleukin-1β cytokine from the platelets (Assinger et al. 2014). CMV-induced TLR2 activation was confirmed by antigenic inhibition and could be blocked by inhibiting phosphoinositide 3-kinase signaling. Vascular endothelial-derived growth factor (VEGF) is a proangiogenic cytokine that is released by TLR2-induced platelet activation and involved in endothelial migration, proliferation, and increased vascular permeability (Assinger et al. 2014). To add further to the many links reported between CMV and vascular disease (Al-Ghamdi 2012; Bruggeman 2000), this TLR2-mediated VEGF release can allow leukocyte recruitment to an atherosclerotic plaque, promoting growth (Holm et al. 2009).

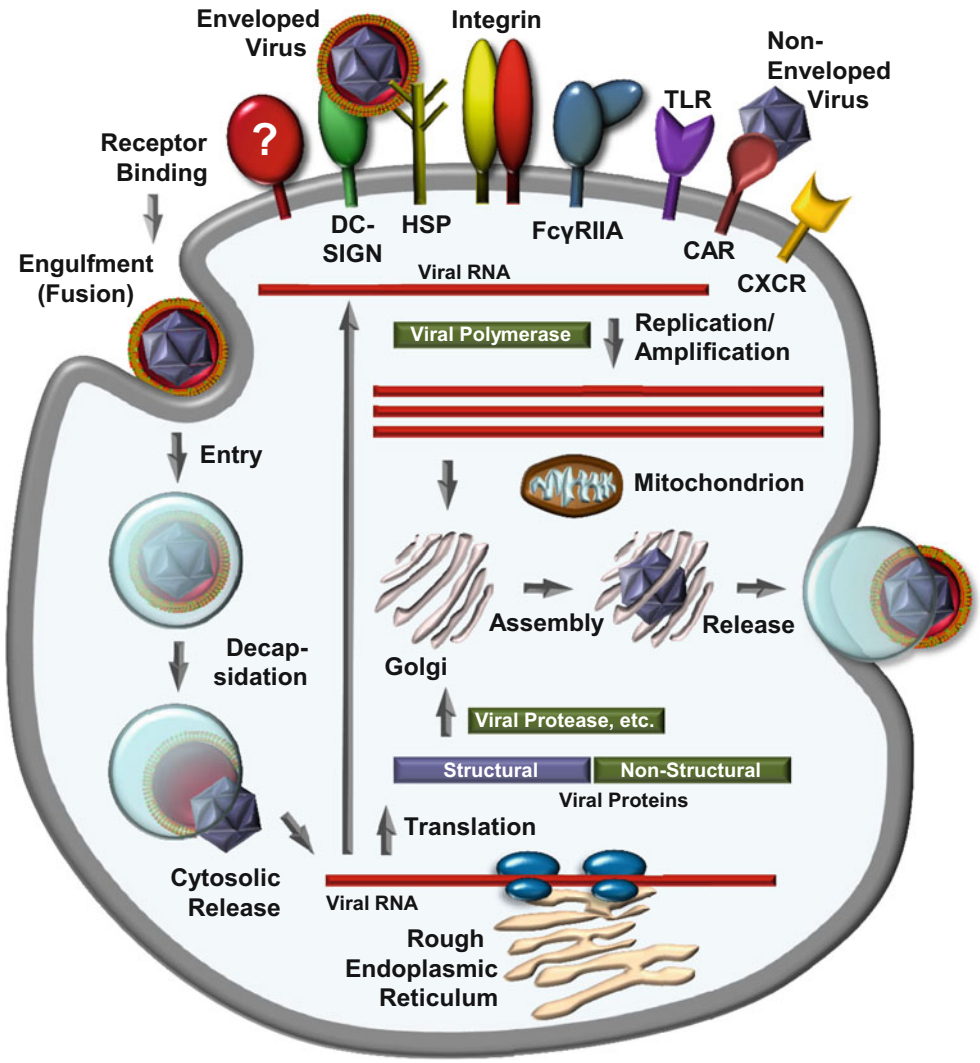

Fig. 1 Virus replication by platelets. Examples of virus receptors present on the surface of the platelet are shown spanning the platelet membrane. Examples of an enveloped virus (DENV binding to the co-receptors DC-SIGN and HSP) and a non-enveloped virus (adenovirus binding to CAR) are depicted. This receptor engagement triggers mechanisms that can involve dynamin-dependent engulfment processes, leading to entry into the platelet as an endosomal inclusion. At least for DENV, which has an RNA genome unlike adenoviruses, evidence is accumulating that suggests viral genomic material is released from the nucleocapsid into the cytoplasm, where it is translated by platelet ribosomal complexes or used as a template for replication by the virus-encoded polymerase. Other virus-encoded genes contribute to controlling the function of the cell or post-translational modification of the viral proteins, including proteolytic maturation if the viral genome is organized to produce a polyprotein. The viral structural proteins and genome copies are then transported to the platelet Golgi apparatus, where they are assembled and delivered to the exterior by exosomal transport or possibly by cell disruption (not shown). Platelet mitochondria are available for energy-demanding aspects of the mechanism

Integrins Consisting of a broad group of homologous heterodimeric proteins (Bennett et al. 2009), cell surface integrins are fundamental to the important platelet–leukocyte connection. In addition to the role of integrins in facilitating numerous direct platelet–virus interactions (Tables 1, 2, and 3), they are also important in processes leading to immune clearance of blood-borne viruses. One example is through the recruitment of dendritic cells by the interaction of platelet surface junctional adhesion molecule-C (JAM-C) and dendritic cell integrin $\alpha_M\beta_2$ (Langer et al. 2007). Trafficking of cytotoxic T lymphocytes (CTLs) to sites of infection can be mediated by platelet activation (Iannacone et al. 2005). In the case of hepatitis B virus (HBV), CTLs enter the liver parenchyma and accumulate where HBV may reside and replicate. CTL activity lowers the virus count, but unfortunately contributes to progression of liver damage (Iannacone et al. 2005).

The HIV-1 transactivator of transcription protein (Tat) directly interacts with platelets, resulting in their activation and degranulation (Wang et al. 2011). This mechanism requires the β3 integrin and chemokine receptor CCR3 to be expressed on the platelet surface. This involvement of integrins was unambiguously demonstrated using a β3

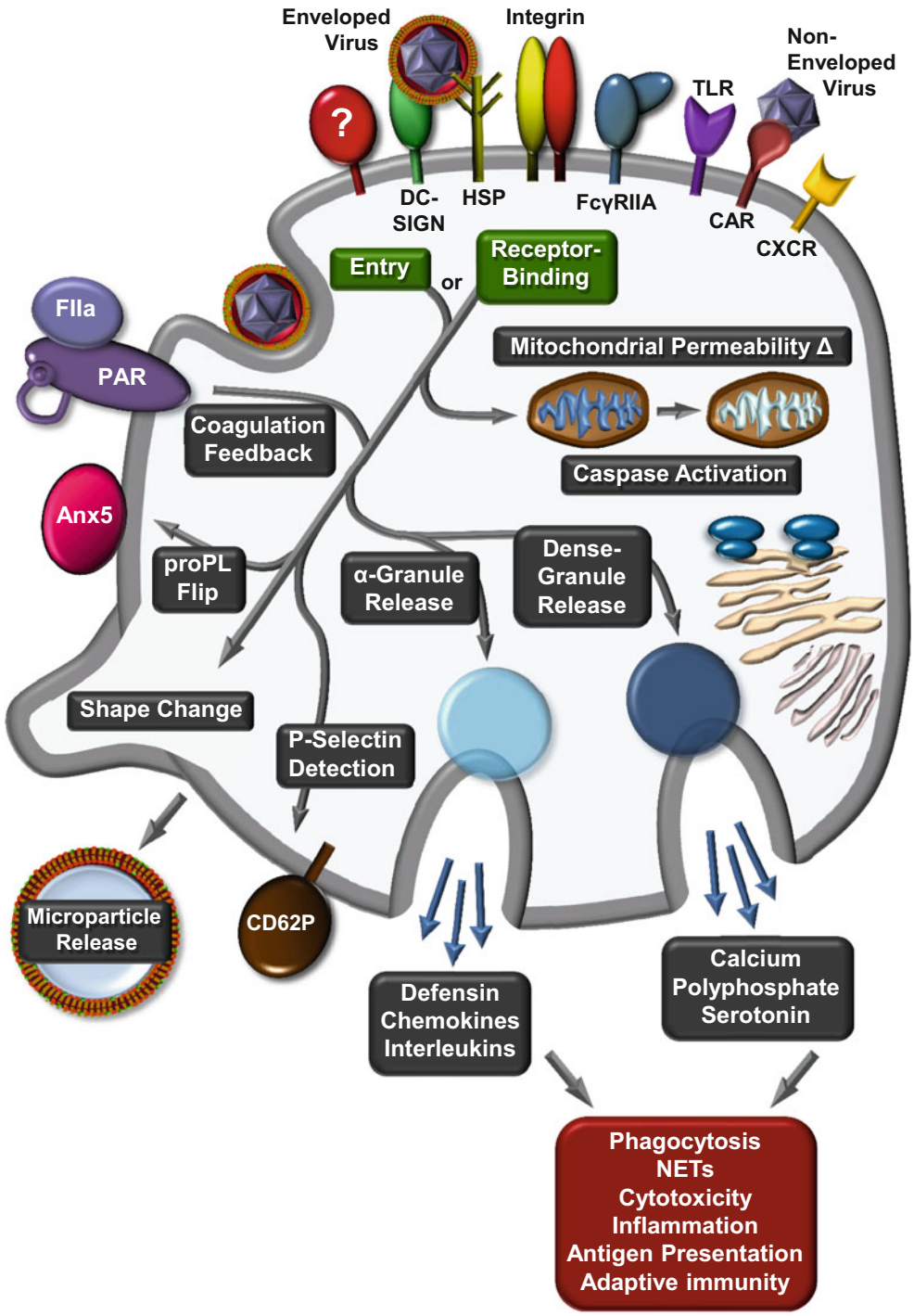

Fig. 2 Virus-induced platelet modulation leading to immune clearance. Platelet receptor binding or possibly entry of the virus induces outside-in platelet signaling, with morphological and biochemical effects to the platelet. Typical platelet "activation" markers are expressed, such as exposure of procoagulant phospholipid (proPL) as measured by annexin V (Anx5) binding and P-selectin (CD62P). Platelet microparticles may be released. The proPL surface can propagate the hypercoagulable state often induced by some of these viruses by enabling assembly of clotting enzyme complexes, where neighboring platelet surface PARs can be activated by thrombin (FIIa) in a feedback-amplified manner to further promote platelet modulation. Virus–platelet engagement has been shown to induce caspase activation and mitochondrial membrane potential changes indicative of an apoptotic state, which similarly occurs for megakaryocytes (not shown) with consequent reduction in thrombopoeisis. Virus-induced platelet stimulation causes the release of α-granule and dense-granule contents. This has profound stimulatory effects on all cells of the immune system, orchestrating localized innate and adaptive responses against virus invasion

knockout mouse model (Wang et al. 2011). Virus-induced platelet degranulation involves the release of proinflammatory CD40 ligand, which promotes platelet–monocyte complex formation via platelet P-selectin and monocyte P-selectin glycoprotein ligand-1 (PSGL-1) (Singh et al. 2014). This was demonstrated by injecting wild-type mice with recombinant soluble CD40 ligand and analyzing cell associations by flow cytometry. The platelet–monocyte complexes derived from HIV-1 infected donors exhibited enhanced adhesion to human brain microvascular endothelial cells, suggesting a role for platelets in HIV-associated neuroinflammation (Singh et al. 2014).

Selectins Although not as extensive as integrins, selectins are also a family of cellular adhesion receptors. Selectins recognize various carbohydrate structures found on opposing surfaces. Platelets constitutively store P-selectin (i.e., CD62P) in α-granules. In response to stimulus-induced activation, P-selectin is transported to the platelet surface where it mediates tethering to numerous cell types. Important to the role of platelets in orchestrating the immune response, platelet surface P-selectin mediates adhesion through association with PSGL-1 found on the neutrophil surface. P-selectin on platelets also interacts with PSGL-1 on a subset of Th1 leukocytes. Thus, when binding of viruses to the platelet surface triggers platelet activation and P-selectin expression on the surface, innate immune clearance of the pathogen is facilitated.

Protease-Activated Receptors Hemostasis and inflammation are regulated and linked by protease-activated receptors (PARs) (Rothmeier and Ruf 2012). Four PAR types have been identified and are implicated both in virus replication (Aerts et al. 2013; Khoufache et al. 2013; Sutherland et al. 2012) and in the host innate response to viral infection (Antoniak et al. 2013). Enveloped viruses can assemble and activate coagulation enzyme complexes directly on their surface, which mediate PAR stimulation. Through these pathways, PAR1 and PAR2 on human umbilical vein endothelial cells have been shown to enhance HSV1 infection in vitro (Pryzdial et al. 2014). A similar mechanism may stimulate virus-bound platelets, which express high levels of PAR1. A more recent study showed that IAV induced platelet activation and aggregation through platelet-surface PAR, which exacerbated acute lung injury (Le et al. 2015). This injury was attributed to the resulting massive inflammation through platelet-induced recruitment of neutrophils to the lung.

Chemokine Receptors Chemokine receptors are members of the G-protein-coupled receptor family, whose major functions include cellular recruitment via chemokine recognition. Several chemokine receptors (CCR1, CCR3, CCR4, CXCR1, and CXCR4) bind to select ligands, resulting in enhancement but not initiation of inflammatory pathways, platelet aggregation, hemostasis, and thrombosis (Zarbock et al. 2007). Activated platelets also secrete numerous chemokines, such as CXCL7 and CXCL8. CXCL7 promotes chemotaxis, adhesion to endothelial cells, and degranulation of neutrophils (Schenk et al. 2002), whereas CXCL8 is important in recruitment of neutrophils (Baggiolini et al. 1994). Active CD40L is secreted by platelets in response to stimulation and binds endothelial CD40, eliciting chemokine secretion and increasing the expression of adhesion molecules on the endothelium (Henn et al. 1998).

Platelet α-granule contents have been shown to limit the spread of HIV-1 in co-cultured T cells (Solomon et al. 2013). CXCL4 (platelet activating factor) and CXCL7 are the most abundant chemokines in the α-granules of platelets (Blair and Flaumenhaft 2009). In particular, CXCL4 released by activated platelets binds to HIV-1 major viral envelope glycoprotein, gp120, proximal to the essential CD4-binding site (Auerbach et al. 2012). The resulting steric inhibition reduced HIV-1 infection by 80 % compared with non-activated platelets using a HIV-1-sensitive cell line that uses reporter gene constructs to quantify infection (Solomon et al. 2013). CXCL4 stimulates neutrophil–endothelial cell attachment and also acts as a co-stimulator of TNF in the release of neutrophil secondary granules (Kasper and Petersen 2011), as further means of enhancing localized immune cell influx.

The proinflammatory cytokine interleukin (IL)-1β is synthesized by platelets as a precursor protein and cleaved by caspase-1 to produce an active form that is released in microparticles (Hottz et al. 2013b). DENV2 was shown to induce IL-1β synthesis directly and secretion from platelets by activating the assembly of a nucleotide-binding domain leucine-rich repeat-containing protein (NLRP3) inflammasome, which controls caspase-1 activity (Hottz et al. 2013a). Generally, IL-1β is important in the acute-phase response, where proteins such as C-reactive protein, complement components, and fibrinogen are produced to destroy or contain microbes (Morrell et al. 2014). Although aiding in the immune response, the IL-1β released from platelets during DENV infection contributes to increased endothelial permeability, thrombosis, and dysregulated hemostasis (Hottz et al. 2013a).

Defensins Defensins are cationic antimicrobial peptides that are key elements in the innate immune system. They act on bacteria, enveloped viruses, and non-enveloped viruses. There are many forms of these small 4–5 kDa peptides, with human platelets expressing β-defensins (hBD) 1, 2, and 3 (Kraemer et al. 2011; Tohidnezhad et al. 2011; Tohidnezhad et al. 2012). Immunofluorescence studies in vitro showed that the inclusion of a selective agonist of neutrophils (phorbol 12-myristate 13-acetate) or platelet PAR1 (thrombin receptor agonist peptide 1) induced

secretion of an adhesive complex from neutrophils, identified as a pathogen "snare." The molecular networks were identified as being composed of long uncoiled strands of DNA and were named neutrophil extracellular traps (NETs) (Brinkmann et al. 2004). NET release is induced by β-defensin 1 secreted from activated platelets (Kraemer et al. 2011). Although most NET involvement in pathogen defense has been elucidated using bacteria, super-resolution structured illumination microscopy revealed that HIV-1 virus particles are also captured by NETs (Saitoh et al. 2012). When these entrapped virus particles were extracted, their infectivity was reduced as a result of highly enriched levels of α-defensin and myeloperoxidase within the NETs.

In addition to HIV-1, hBD-2 is also known to inhibit human respiratory syncytial virus (RSV) entry into human lung epithelial cells, as followed by ^{35}S-labeled RSV uptake (Kota et al. 2008). It was also shown through electron microscopy and buoyant density profiles that hBD-2, but not hBD-1, disrupted the viral envelope, possibly because the cationic nature of the peptide led to lipid destabilization (Kota et al. 2008). HIV-1 induced hBD-2 and hBD-3 expression in human oral epithelial cells, which associated directly with HIV-1 and neutralized infection in vitro (Quinones-Mateu et al. 2003). IAV hemagglutinin and hBD-3 binding also resulted in inhibition of epithelial cell infection (Leikina et al. 2005). hBD-3 was shown to alter fusion between IAV, sindbis virus, baculovirus, and synthetic target membrane by crosslinking virus surface proteins (Leikina et al. 2005). Although reasonable to speculate, it is not known whether platelet-derived hBD-2 and hBD-3 mediate similar direct antiviral effects.

Other Secreted Platelet Components Human donor platelet concentrates contain unknown antiviral activity that reduced the viral titer of poliovirus 1, adenovirus 5, and vaccinia virus by approximately 2.63 ± 0.5 to 5.6 ± 0.9 log units (Maurice et al. 2002). The same group also observed platelet activation in all virus–platelet co-cultures with epithelial monolayers (Vero cells); recent knowledge (Flaujac et al. 2010) suggests that platelet releasate is the antimicrobial factor(s) in these studies.

Defensins and cytokines are stored in platelet α-granules. However, dense granule constituents such as adenosine diphosphate (ADP) (Packham and Rand 2011), polyphosphates (polyP) (Smith and Morrissey 2014), and serotonin (Jedlitschky et al. 2012) also have immune-modulating properties (Morrell et al. 2014). These have not been as well characterized for a role in viral innate immunity as those contained in α-granules. Nevertheless, the release of platelet dense granule contents by viruses has been investigated in CMV infection. This was mediated by platelet surface TLR2 occupation. Furthermore, inhibition of the ADP receptor, $P2Y_{12}$, identified ADP release as an important trigger for secondary platelet activation (Assinger et al. 2014). Dendritic cells also express $P2Y_{12}$ and their stimulation increases antigen endocytosis and processing (Vanderstocken et al. 2010), which could involve localized platelet response to pathogen interactions. PolyP induces proinflammatory responses by acting on the nuclear factor κB (NF-κB) pathway in vascular endothelial cells (Bae et al. 2012). PolyP can also interact with chromatin-associated nuclear proteins such as high mobility group box 1 (HMGB1) to amplify proinflammatory responses (Dinarvand et al. 2014). Because HMGB1 is also secreted by platelets (Maugeri et al. 2012), polyP warrants continued study as a bridge between platelets and innate immunity, especially in the context of complement, which is suppressed by polyP (Wat et al. 2014) and could be a viral survival mechanism.

Virus-Induced Thrombocytopenia

According to some estimates, approximately two-thirds of acute thrombocytopenia cases are preceded by viral infection (Rand and Wright 1998). This strikingly high correlation suggests mechanisms directly linking the virus to the platelet, such as receptor-mediated binding (Table 1). In contrast to viral thrombocytopenia, viral HF (Table 2) is thought to be driven by severe suppression of innate immunity and the resulting cytokine flood that counters systemic virus replication. HF can also result in a reduced platelet count, but through mechanisms indirectly involving platelets, as detailed in excellent reviews elsewhere (Feldmann and Geisbert 2011; Messaoudi and Basler 2015; Zapata et al. 2014). Here, we overview five primary pathways that simultaneously contribute to viral thrombocytopenia in the absence of HF: decreased thrombopoeisis, direct and indirect virus interactions with platelets and megakaryocytes (tipping the intricate balance between thrombopoeisis and platelet clearance), altered platelet function, and virus-induced immune response against platelets.

Decreased Thrombopoeisis Viral infection results in production of interferon, which has antiviral activity. However, type I interferons can also inhibit megakaryocytes, resulting in impaired platelet production (Rivadeneyra et al. 2015). Megakaryocyte growth and differentiation is stimulated by thrombopoietin (TPO), which is predominantly produced in the liver (Giannini et al. 2002). Impaired liver function is consequently detrimental to TPO production and the resulting thrombocytopenia often suffered by chronic HCV patients correlates with low TPO and attenuated thrombopoeisis (Giannini et al. 2002; Wenzel et al. 2010).

Direct impairment of platelet production as a result of viral replication in megakaryocytes was observed in vitro for HIV, HCV, DENV, and CMV (Basu et al. 2008; Crapnell et al. 2000; Li et al. 1999; Sato et al. 2000; Sridharan et al. 2013). An increase in cell death via apoptosis and decreased megakaryocyte differentiation are probable reasons for reduced platelet production. For example, HIV infection of megakaryocytes resulted in downregulation of the TPO receptor c-Mpl (Gibellini et al. 2013), causing reduced sensitivity to thrombopoeisis induction. To further investigate this mechanism, umbilical cord blood hematopoietic progenitor cells that were induced by TPO toward a megakaryocytic lineage in vitro have been used as a model for HIV infection. The virus surface glycoprotein gp120 interacts with CD4 on these cells (Gibellini et al. 2007), resulting in specific protein and mRNA changes that elevate TGF-β1 levels and decrease tumor necrosis factor (ligand) superfamily, member 13 (TNFSF13) levels. Both of these effects contribute to inhibition of megakaryocytic proliferation and promote apoptosis (Gibellini et al. 2007). Further evidence supporting the concept that viral thrombocytopenia involves attenuated thrombopoiesis comes from an elegant humanized mouse model showing that DENV infection reduces platelet production (Sridharan et al. 2013). Thus, viral infection has the capacity to reduce thrombopoeisis by direct effects on the megakaryocyte, conceivably involving the same surface receptors for specific viruses that have been identified on platelets and other cells (Tables 1, 2, and 3).

Direct Virus–Platelet Associations Activation of platelets, degranulation, and recruitment of neutrophils and dendritic cells contribute to phagocytosis of platelet fragments by leukocytes. The final clearance of these potentially virus-laden particles is in the spleen and liver (Bondanza et al. 2001; Grozovsky et al. 2010; Koupenova et al. 2014). CMV- or EMCV-mediated activation of platelets via TLR2 or TLR7, respectively, enhanced the interaction between platelets and neutrophils, resulting in platelet clearance (Assinger et al. 2014; Koupenova et al. 2014). In a similar manner, hantavirus also induced platelet activation and clearance by binding to platelet surface integrins $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (Gavrilovskaya et al. 2010). The interaction of hantavirus with platelets also contributed to viral dissemination and activation of endothelial cell functions, thereby increasing vascular permeability (Feldmann and Geisbert 2011; Gavrilovskaya et al. 2010).

Adenovirus correspondingly induces thrombocytopenia, as seen when adenovirus gene therapy vectors are intravenously administered to rhesus macaques and mice (Othman et al. 2007; Wolins et al. 2003). Current literature indicates that CAR mediates the binding of adenovirus to platelets, enabling subsequent entry (Othman et al. 2007; Shimony et al. 2009). The virus–platelet interaction is predominantly localized to sites of intercellular complex formation, implying that CAR expression is enhanced in response to platelet activation (Gupalo et al. 2011). Similar to the effects reported for other viruses, platelet activation is probable upon the initial association of adenovirus and platelet-surface CAR.

The IAV envelope has neuraminidase activity that cleaves sialic acid on the surface of platelets (Madoff et al. 1964). Removal of more than 15 % of total sialic acid on rabbit platelets caused complete platelet clearance within 1 h of administering ^{51}Cr-labeled platelets into rabbits (Greenberg et al. 1975). The removal of circulating platelets was presumably caused by recognition of exposed terminal galactose residues by scavenger cells expressing the asialoglycoprotein receptor (Sorensen et al. 2009). This adds to the repertoire of virus-clearance mechanisms that can be facilitated through direct virus–platelet association.

Indirect Virus–Platelet Associations Platelets express Fc receptors that allow recognition of immune complexes or aggregated immunoglobulin. In addition to PAR-1 activation by thrombin, IAV can activate platelets through low-affinity FcγRIIA signaling (Boilard et al. 2014). For this to occur, IAV must be decorated with anti-IAV IgG. Crossreactive antibodies resulting from immune recognition of different IAV strains (H1N1 versus H3N2) was sufficient to produce this effect. When wild-type mice (which do not express FcγRIIA) and transgenic mice expressing human FcγRIIA were challenged intravenously with a sublethal dose of H1N1, there was a drop in circulating platelet count in only the transgenic mice. This indicates that platelet homeostasis is affected by the accessibility of Fc receptors on platelets, supporting the link to pathogenic thrombocytopenia (Boilard et al. 2014).

Activation of endothelial cells has been implicated in adenovirus-induced thrombocytopenia. In these studies, the release of ultralarge von Willebrand factor (vWF) from the Weibel–Palade bodies (Gupalo et al. 2011) was evaluated using a murine model. Adenovirus induced thrombocytopenia in wild-type animals but, in sharp contrast, vWF knockout mice were protected from a reduced platelet count (Gupalo et al. 2011). Thus, endothelial vWF could contribute in an indirect manner to virus-induced thrombocytopenia by supporting platelet aggregation through interactions with platelet surface GP1b, (the vWF receptor). Clearance of these platelet aggregates is subsequently facilitated by splenic macrophages or Kupffer cells in the liver (Othman et al. 2007).

Systemic inflammation as a result of viral infection can result in platelet interactions and increased clearance, as demonstrated during IAV, rhinovirus, and CMV infections (Bouwman et al. 2002). The mechanism involves mononuclear leukocytic release of CXCL4. DENV infection was

used in a subsequent study and resulted in increased inflammation, vascular permeability, and platelet aggregation and activation (Yang et al. 1995).

Altered Platelet Function Thrombocytopenia can result from virus activation of platelets or endothelial cells, which induces cell–cell adhesion processes via expression of integrin and selectin function (Zapata et al. 2014). The flipside is that inhibition of platelet aggregation can also result in impaired platelet function. DENV infections have been shown to stimulate platelet-directed IgM autoantibodies that inhibit ADP-induced platelet aggregation (Lin et al. 2001). Although these autoantibodies have an effect on immune clearance of platelets, they also affect the development of HF. For hantavirus, this can include renal syndrome resulting from defective platelet aggregation (Cosgriff et al. 1991). Other HF viruses such as Lassa virus, Junin virus, and Ebola virus have inhibitors of platelet aggregation, but these have not been identified (Cummins et al. 1989; Cummins et al. 1990, Feldmann and Klenk 1996). Additionally, vaccinia virus causes impairment of platelet aggregation induced by ADP, collagen, or thrombin (Bik et al. 1982). Ebola virus infection also results in increased levels of type I interferons, which downregulate platelet production and function (Rivadeneyra et al. 2015; Villinger et al. 1999).

Virus-Induced Immune Response Against Platelets The molecular relationship between autoantibodies against platelet proteins and viral infection is complex and not yet clearly understood. Virus-induced thrombocytopenia typically worsens as damage to the liver progresses and can deteriorate into a more severe clinical complication (Aref et al. 2009). Anti-platelet autoantibodies are linked to immune thrombocytopenic purpura (ITP) (Liebman 2008). Secondary ITP can result from vaccines such as measles-mumps-rubella (MMR) (incidence of 1 in 40,000 administrations) or infections with homologous herpes family viruses, hepatitis C, HIV, hantavirus, and severe acute respiratory syndrome coronavirus (Goeijenbier et al. 2012; Liebman 2008).

Non-AIDS early HIV-1 infections can result in ITP induced by autoimmune antibodies. Affinity purification of circulating serum immune complexes in HIV patients identified an anti-HIV IgG1 antibody that recognizes amino acid residues 49–66 of the integrin β3 subunit that induces platelet vessiculation (Nardi et al. 2001). The complement pathway was ruled out in this reaction because neither the $F(ab')_2$ fragment of the antibody raised against integrin β3 residues 49–66 in wild-type mice nor treatment with full-length antibody in C3-deficient mice could affect platelet microparticle formation (Nardi et al. 2001). Peroxide generation was monitored through the use of an intracellular fluorescent probe and revealed a novel mechanism by which the autoantibody induced damage in platelets through a NADPH oxidase peroxide-generating pathway (Nardi et al. 2001). Similar outside-in signaling events could be generated by other virus-induced platelet autoantibodies.

ITP occurs in 20 % of HCV patients and is potentially attributed to the presence of antibodies that are crossreactive with HCV core envelope 1 protein and platelet β3 integrin (Rajan et al. 2005; Zhang et al. 2009). The crossreactivity was found by using the antibody specific for integrin β3 residues 49–66 to screen a phage-display peptide library (Zhang et al. 2009). The recognized peptides were then aligned with the viral genome to define similarities. The matched peptides were rationally designed as tools to inhibit the binding of autoantibodies to platelets or to produce platelet antibodies with functional effects (Li et al. 2005; Zhang et al. 2009). This approach was similarly used to discover molecular mimics in the HIV-1-encoded negative regulatory factor (nef) protein (Li et al. 2005), both leading toward therapeutic design in virus-induced thrombocytopenia.

Antibody crossreactivity between antiviral antigens and platelet antigens has been demonstrated during DENV infection (Cheng et al. 2009). Pairwise sequence alignment analysis programs were used to annotate homologous peptide sequences between DENV NS1 and platelet protein disulfide isomerase (PDI) (Cheng et al. 2009). This tool identified several sequence homologies, of which amino acid residues 311–330 (P311–330) was the most dominant epitope recognized by both anti-NS1 and anti-PDI, as determined by ELISA (Cheng et al. 2009). The P311–330 antibodies generated from hyperimmunized mice bound platelet PDI and inhibited both thiol isomerase activity and platelet aggregation induced by ADP (Cheng et al. 2009). The presence of anti-platelet autoantibodies with anti-DENV activity in DENV patient sera is associated with thrombocytopenia and the severity of the disease during the acute phase of secondary DENV infection (Saito et al. 2004). However, the direct implication of these crossreactive antibodies in DENV pathogenesis has not yet been elucidated. The discovery that platelets can translate and express NS1 (Simon et al. 2015) suggests that viral antibodies might not be crossreactive with platelets, but actually recognize the virus-encoded gene product expressed on the platelet, further complicating vaccine development.

Conclusion

Associations between viruses and platelets can lead to pathology. Viral infection often precedes thrombocytopenia and, therefore, an understanding of the mechanisms that facilitate virus–platelet interactions, their direct effect on platelets, and indirect effects on the cellular environment can lead to therapeutic control. This is a difficult challenge because platelets also help to eradicate viruses by steering

innate and adaptive immune responses. Thus, ideal therapeutic control of virus-induced thrombocytopenia can discretely manage both the detrimental and positive involvement of platelets in viral infection.

Take Home Messages

- Diverse virus families can bind to platelets, resulting in mild to severe clinical outcomes.
- Virus–platelet interplay results in changes to innate and adaptive immunity.
- Platelets replicate the RNA genome of permissive viruses, which can contribute to pathology.

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Platelets in Malarial Infection: Protective or Pathological?

Simon Foote, Gaétan Burgio, and Brendan McMorran

Abstract

Platelets play an ambiguous role in a malarial infection. On the one hand, platelets have been implicated adversely in cerebral malaria. They stick to the cerebral endothelium and mediate the adhesion of infected erythrocytes, with the postulated outcome of increasing severity or of even mediating this disease. On the other hand, platelets bind to infected red cells in the periphery and activate and release the cytokine PF4. This, in turn, binds to the Duffy antigen on the red cell and is thought to be internalised and enters the parasite food vacuole which it then destroys, killing the parasites. The control of platelet levels during an infection is also discussed with a view to understanding the thrombocytopenia that frequently accompanies a malarial infection.

Malaria is a haematological disease. All its clinical consequences are due to its erythrocytic life stage. Therefore, given its intimate sharing of a biological niche with platelets, it is not surprising that the parasite, living within the red cell, interacts with the platelet. Malarial parasites are single cellular eukaryotic organisms that are introduced into the host through the bite of an infected Anopheles mosquito. These sporozoites travel to the liver where they undergo many rounds of replication and, upon erupting from the hepatocyte as small merozoites, infect red cells. Their lifecycle in the host is thereafter centred on the red cell. Rounds of erythrocytic invasion, replication and lysis continue until the host develops an adaptive immune response that kills all parasites. An effective immune response against malarial parasites takes years to develop as the parasite population houses a staggering suite of antigenic variation. The lifecycle is continued by the development of dimorphic sexual stages (gametocytes) that complete the lifecycle in the mosquito after ingestion by a mosquito. Gametocytes undergo meiosis in the mosquito. This produces recombinant, segregated parental chromosomes in the progeny. If the mosquito ingests more than one type of parasite, sporozoites will be produced with different combinations of variant antigens resulting in an immunologically unrecognisable parasite to hosts previously infected with either parental parasites.

Platelets are known to be involved in all but the hepatic stage of infection. They are inhibited during mosquito feeding and removed during the erythrocytic stages. They appear to both ameliorate infection by killing parasites and compromise the host by mediating adhesion of infected erythrocytes to the vascular wall. These various roles of the platelet will be discussed in detail in this chapter.

Platelet Inhibition by Feeding Mosquitoes

The saliva from the female *Anopheles stephensi* contains many compounds that prevent the clotting of blood, allowing the smooth feeding of the mosquito. It is during this feeding that malarial sporozoites are injected into the host. The anopheline antiplatelet protein (AAPP) has been isolated from mosquito saliva and has significant inhibitory effects in the prevention of platelet aggregation in the presence of collagen (Yoshida et al. 2008). It inhibits binding of platelets to collagen as well as inhibiting platelet-platelet binding. When used

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as a candidate antiplatelet agent in mice, it ameliorated a mouse thrombosis model without excessively increasing the bleeding time. Its antiplatelet activity compared well to that of aspirin (Hayashi et al. 2012).

Thrombocytopenia Is a Feature of a Malarial Infection

A constant companion to a malarial infection is a decrease in parasite density. This thrombocytopenia was seen early in rodent infection experiments in the 1950s (Fabiani et al. 1958), but has been regularly (and frequently) reported over the decades since. Thrombocytopenia is fairly consistently seen in models of human malaria; thus, it is seen in hamsters infected with *Plasmodium berghei*, a rodent malaria (Essien et al. 1984), in *P. vinckei*-infected mice (Reiner et al. 1991) and *P. chabaudi*-infected mice (McMorran et al. 2009) (Fig. 1) and is quite severe in *P. knowlesi*-infected macaque monkeys (Abildgaard et al. 1975).

There are four major human malarias, *P. ovale*, *P. vivax*, *P. malariae* and *P. falciparum*. Thrombocytopenia is a common feature of all these malarias (Chimalizeni et al. 2010; Dennis et al. 1967; Essien 1989; Jeremiah and Uko 2007; Kehinde et al. 2005; Ladhani et al. 2002; Leowattana et al. 2010; Paul 1990; Shaikh et al. 2011; Soupison et al. 1995; Spitz 1946; Taylor et al. 2008; Touze et al. 1990). However it is *P. falciparum* and *P. vivax* that are the most extensively analysed. Patients with *P. vivax* infections have anecdotally lower platelet counts, epitomised by a study from Papua where both *P. falciparum*- and *P. vivax*-infected trial enrollees had their platelets measured. Significantly lower levels were associated with *P. vivax* infection (Taylor et al. 2008).

The proportion of malaria-infected patients with thrombocytopenia varies from study to study (Chimalizeni et al. 2010; Gerardin et al. 2002; Ladhani et al. 2002). However, there is little doubt that the majority of patients with malaria show some level of thrombocytopenia. Percentages range from close to 100 % of all malaria-infected individuals being thrombocytopenic, in an early study in Singapore (Beale et al. 1972), to 85 % of infected children in India (Prasad et al. 2009) and to 56 % of children in Kenya (Ladhani et al. 2002). Moreover, platelet numbers are decreased in asymptomatic as well as symptomatic malaria (Jeremiah and Uko 2007; Kehinde et al. 2005).

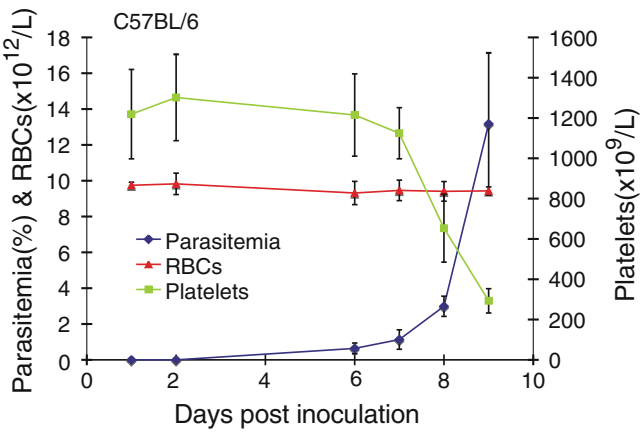

Fig. 1 Platelet, parasite and RBC kinetics in a *P. chabaudi* infection in mice. The rise of parasitaemia is directly reflected in a fall in platelets at a time early in infection when the red cell number remains unchanged

Clinical Corollaries of Malarial Thrombocytopenia

Early reports associated the severity of malarial thrombocytopenia from falciparum malaria with severe malaria (Gassner et al. 1979). This has been corroborated by more recent reports (Gerardin et al. 2002) where severe malaria and thrombocytopenia are linked and where thrombocytopenia was seen as an independent predictor of death. However, there are other reports showing that there is no direct link between the level of thrombocytopenia and the severity of malaria (Chimalizeni et al. 2010; Ladhani et al. 2002). The most reliable association of thrombocytopenia is with the level of parasitaemia, with an inverse correlation seen between platelet and parasite densities. In a study in Malawi, 1811 children with cerebral malaria were compared with 521 children with bacterial meningitis. The children with malaria had fewer platelets, but, again, there was no correlation between the level of thrombocytopenia and parasite density. What is true for severe malaria is also true for asymptomatic malaria (Jeremiah and Uko 2007; Kehinde et al. 2005) where the correlation between thrombocytopenia and parasite density is not great. So, it would appear that if there is an association with severe malaria that is independent of parasitaemia, then its effect is not large.

The second possible clinical correlate of a malaria-associated thrombocytopenia is the presence of a propensity to bleed. However, bleeding is not common in malarial infections. Studies looking at clotting parameters report conflicting results with abnormalities in prothrombin time, activated thromboplastin time and thrombin time (Prasad et al. 2009). However, these changes were not seen in another study reported by Mohanty et al. (1988). Nonetheless, abnormalities in platelet function were observed by Prasad et al. who reported hypoaggregation of platelets with ADP and ADR in greater than 90 % of all cases (Prasad et al. 2009), although there were very few examples of bleeding. Bleeding has been reported however in severe cases of malaria with frequencies above 5 % (Srichaikul 1993) and is seen at frequencies of 55 % in autopsy cases (Boonpucknavig et al. 1984).

The most common site of haemorrhage is seen in the retinas of children with malaria, part of the malaria retinopathy, which is a quartet of changes common in cases of malaria (Beare et al. 2006, 2009; Lewallen et al. 2008).

The relationship between retinal haemorrhage and clotting deficiencies and/or platelet changes has not been studied.

Cause of Malarial Thrombocytopenia

A loss of platelets during malarial infection may be caused by an increase in the consumption or destruction of platelets, or by a decrease in their production, or by a combination of both. That there might be a decrease in the production of platelets is based on the observation that erythropoiesis is suppressed by malarial parasites (Miller et al. 1989; Mohan and Stevenson 1998; Yap and Stevenson 1991). However examination of the bone marrow from thrombocytopenic malaria-infected patients showed no decrease in the number of megakaryocytes and, indeed, a megakaryocytosis in one patient (Beale et al. 1972). Despite recent studies appearing to exonerate thrombopoiesis from malaria-mediated suppression (de Mast et al. 2010), Casals-Pascual and colleagues showed in a large cohort of children infected with malaria, and with thrombocytopenia, that the level of platelets was inversely correlated with circulating IL10 levels (Casals-Pascual et al. 2006). They pointed to the known thrombocytopenic effect of IL10 administration caused by a decrease in megakaryocytosis (Sosman et al. 2000) could be also causing the thrombocytopenia seen in malaria patients with raised IL10.

There have been many suggestions as to why platelets are being cleared during malarial infection; these include disseminated intravascular coagulation (DIC), immune-mediated destruction, pooling within the reticuloendothelial system, sequestration in the microcirculation and malaria-mediated apoptosis. Early studies reported DIC as a cause of thrombocytopenia (Jaroonvesama 1972; Spitz 1946), and these observations resulted in the use of heparin in severe malaria. However, these observations have not been validated, and there is now significant data to show that DIC is not common in malaria (Beale et al. 1972) and that DIC is not the cause of most malaria-related thrombocytopenias (Skudowitz et al. 1973). There continue, however, to be isolated reports of DIC in end-stage severe malaria cases, so it is possible that DIC is a rare complication of late-stage malarial infections (Gassner et al. 1979; Pukrittayakamee et al. 1989; Reid and Nkrumah 1972). Given that platelet number begins to decrease early in infection, it is unlikely that this is due to DIC.

Immune-mediated clearance of platelets during malarial infection has been proposed as a mechanism underpinning thrombocytopenia. In a study of 28 patients infected with mainly *P. vivax*, Kelton and colleagues showed that platelet-associated IgG, in the presence of malarial antigens, bound platelets (Kelton et al. 1983). In a single case study, Conte identified platelet-associated IgG and IgM, and antibodies binding to the platelet antigens CD41 and CD49b (Conte et al. 2003). However, there was no evidence given that these were responsible for the thrombocytopenia. In a similarly small sample from Japan, platelet-associated antibodies were found in two patients with *vivax* malaria (Yamaguchi et al. 1997). The common thread linking these different studies is that upon treatment with antimalarial drugs, the binding of either IgG or IgM disappeared. This is inconsistent with a hypothesis of autoimmune antiplatelet antibodies, as, presumably, these would persist some time after the malarial infection was cleared. This phenomenon appears to more resemble heparin-induced thrombocytopenia where antibodies directed against PF4-heparin complexes are captured by platelet Fc receptors, causing the activation and removal of platelets (Kasthuri et al. 2012). This disease is stopped when heparin administration is ceased. In a similar fashion, the binding of IgG to platelets in malarial infection requires the presence of malarial antigens and stops as soon as antigen is withdrawn upon successful treatment of disease.

Malaria is often accompanied by splenomegaly. The increase in splenic size is due to an accumulation of macrophages, and these phagocytose both infected and noninfected red cells (Russell et al. 1946). There have been suggestions that platelets pool in these large spleens and are phagocytosed by the reticuloendothelial system (Skudowitz et al. 1973). However, there is no correlation between splenic size and platelet density (Beale et al. 1972). Karanikas and colleagues performed scintography on radiolabelled platelets in normal and malaria-infected patients. They did not find pooling in the spleen, even in individuals with very large spleens. In fact the distribution of platelets was indistinguishable from control individuals. They did however see a greatly reduced platelet half-life (Karanikas et al. 2004). Sodeman et al. infected rhesus monkeys with *P. cynomolgi* and saw no differences in platelet number between splenectomised and intact animals. These data would tend to rule out the reticuloendothelial system in playing a large role in the clearance of platelets in malarial infection.

Platelet death can be mediated through a process analogous to apoptosis in nucleated cells. This is initiated through activation of platelet caspases. In a mouse model of malaria, caspases have been shown to be activated causing platelet apoptosis (Piguet et al. 2002). This was associated with an increase in the number of circulating platelet microparticles, a possible corollary of platelet apoptosis. The thrombocytopenia in these mice could be decreased with pretreatment with anti-CD40L antibodies or with caspase inhibitors, indicating that apoptosis was initiated by CD40L and that caspases were crucial.

The Role of Platelets in the Pathophysiology of the Malarial Infection

Platelets may modulate the clinical consequences of malarial infection through two major routes and perhaps a number of less well-studied pathways. These include multiple

observations that platelets, through their ability to bind both infected and noninfected red cells and the endothelium, may be the major mediator of cerebral malaria. Alternatively, and not exclusively, platelets have been shown to bind to infected red cells and to kill the parasites within. While both these mechanisms appear contradictory, they may both be active in a malarial infection. However, they both rely on an important attribute of the platelet: its ability to adhere to both the red cell and the endothelium.

Cytoadherence of Platelets During Malarial Infection

Platelets bind to infected red cells in both mouse and human malaria. The binding molecules on the platelet side of this interaction have been well studied and include CD36, ICAM1 and gC1qR (Mayor et al. 2011). The adhesion phenomenon is seen as platelet-mediated rosetting, clumping or infected red cell binding to purified receptors. Infected red cells also bind to the endothelium, and it is through this sequestration from the reticuloendothelial system that parasites are able to reproduce more effectively. Given that some of the receptors that bind infected red cells to platelets are also present on the endothelium of the microcirculation, there is the possibility that adhesion to platelets is a side effect of the development of endothelial binding by infected red cells to avoid splenic clearance.

Binding of Platelets to Infected Red Cells and Endothelium and the Development of Cerebral Malaria

Cerebral malaria, along with severe malaria anaemia, is the major mechanism of mortality in patients infected with *P. falciparum*. The exact pathophysiology of cerebral malaria has yet to be elucidated. However, the disease is characterised by a high mortality rate of at least 20 % and by a profound coma from which most people who survive do so with little or no neurological sequelae, although a small percentage do go on to retain a neurological deficit. The pathology appears to involve the physical binding of infected red cells to the endothelial cells of small vessels in the brain. This is usual in many organs, but in the brain it produces cerebral malaria. There are also immunological causes to the syndrome which may explain the majority of the pathology as a mechanical cause due to obstruction alone, but this is not consistent with the excellent outcome in most surviving patients.

Platelets must be involved however as early observations reported that platelets are seen in plugs in cerebral blood vessels. In an electron microscopic study looking at the brains of malarial patients who had died from *P. falciparum*-induced cerebral malaria, abnormal infected red cells with surface knobs were seen binding to endothelium. Although there were some small number of uninfected cells, most were infected, and some of these lesions contained platelets (Pongponratn et al. 1985). This observation has prompted the hypothesis that cerebral malaria is caused by the binding of platelets to infected red cells and that this mediates their adhesion to the endothelial surface (Biswas et al. 2007). In addition, platelets may activate and release mediators for the immune response that ensues. However, more recent, electron ultrastructural studies have not been able to repeat these early findings. So, in 2003 a study looking at a large number of children who had died from cerebral malaria found that only 6 % of all cerebral lesions contained platelets (Pongponratn et al. 2003). This would indicate that, perhaps, platelets do not play an important role in cerebral malaria than had been thought.

Unfortunately there is no perfect animal model for cerebral malaria (White et al. 2010). The model that is most published is the murine malaria, *P. berghei*, which produces a cerebral malaria-type syndrome early in the infection in susceptible mouse strains. However the pathophysiology is quite different from human cerebral malaria. The lesions seen with most *P. berghei*-infected mice involve very few infected cells, and most of the lesions contain uninfected red cells and large mononuclear phagocytic cells. This is very different from human cerebral malaria where the large mononuclear cells are absent, and the lesions contain very few uninfected cells. It is, however, possible that there are some shared effector pathways between the human and mouse cerebral malaria (Grau and Craig 2012).

There is a burgeoning literature implicating platelets in the pathophysiology of *berghei* malaria in mice. Mice infected with the murine parasite *P. berghei* develop cerebral malaria early in the infection, but only in strains of mice susceptible to disease. Platelets have been implicated in the pathology of this disease from several types of evidence; platelet binding to the endothelium of cerebral microvessels is increased in *P. berghei*-infected mice, and anti-C41 antibody depletion of platelets early in the infection prevents cerebral malaria (Sun et al. 2003; van der Heyde et al. 2005). The observation that either ICAM-1 or P-selectin deficiency also prevents the development of cerebral malaria would indicate that platelets play a role and that binding to the endothelium via either ICAM-1 or P-selectin is part of that role (Sun et al. 2003). However, mice with platelets cleared one or two days before the onset of cerebral malaria were not protected (van der Heyde et al. 2005), which would counter the argument that adherent platelets play a role in the development of cerebral malaria as there were no platelets present in these mice, and yet the development of cerebral malaria continued unabated.

Further evidence that platelets play a role in cerebral malaria comes from the ameliorating effects on the development of cerebral malaria in mice deficient for the platelet protein, platelet factor 4 (PF4) or its chemokine receptor CXCR3 (Srivastava et al. 2008). Prevention of activation of platelets with aspirin also decreased the severity of cerebral malaria in the *berghei* model (Srivastava et al. 2008). That platelet-derived PF4 might be playing a more complex role in the development of cerebral malaria is suggested by studies that show that in animals PF4 drives monocyte cytokine production and that depletion of monocytes gives rise to resistance to cerebral malaria in the *berghei* model. Furthermore, PF4-deficient mice show less trafficking of monocytes to the brain, a PF4 effect mediated through expression of KLF4 (Srivastava et al. 2010). In support of these findings is the observation that mice, either lacking the platelet-activating factor (PAF) receptor or being treated with a PAF receptor antagonist, have an ameliorated response to cerebral malaria (Lacerda-Queiroz et al. 2012).

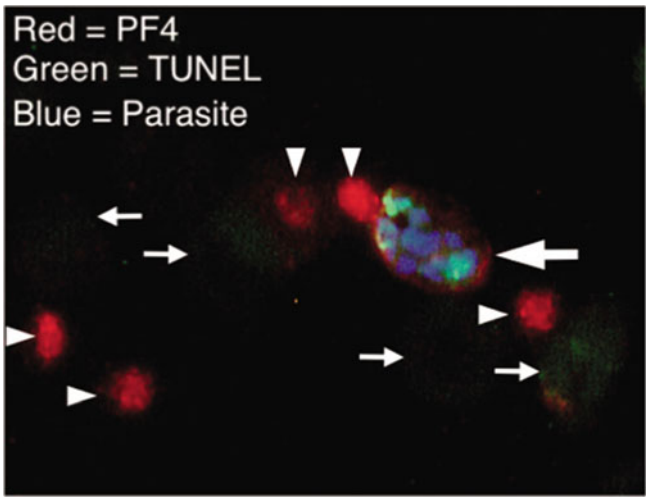

Fig. 2 Immunofluorescent image of platelets binding to both infected and uninfected red cells. Note that PF4 (*red stain*) is only released onto the erythrocyte if there is a parasite within the cell. And also the PF4-coated parasite is Tunel positive, i.e. dead

Platelets May Offer Protection Against the More Severe Effects of a Malarial Infection

Early studies have shown that purified platelets added to a culture of *P. falciparum* inhibit the growth of the parasites (Peyron et al. 1989). This inhibition of growth was only dependent on the presence of the platelets. This observation was replicated nearly a decade later where Polack et al. showed that antibody depletion of platelets resulted in animals that were more susceptible to a *P. berghei* infection than intact animals. McMorran et al. reproduced the mouse findings in an *Mpl* knockout mouse that has only 10 % of the normal number of circulating platelets. These mice had an enhanced survival when infected with the murine malaria, *P. chabaudi*, and their parasitaemia was lower than intact animals. These results were repeated with purified platelets in culture with *P. falciparum*. Both in the mouse and in culture, platelets preferentially bound to infected cells (Fig. 2), although noninfected red cells also bound. Parasites in cells bound to platelets were killed (McMorran et al. 2009). Killing of parasites by platelets required activation of the platelet, and killing was blocked by inhibition of platelet activation by aspirin. Aspirin is a common antipyretic drug in the developing world, and this observation may bring doubt to the wisdom of the continued use of aspirin in malarial infections.

The molecular basis of this killing was PF4, released by activation of platelets upon binding infected red cells. PF4 entered the red cell through the Duffy antigen, which is a chemokine receptor. In most Africans, there is a null allele of the Duffy antigen which is fixed as a homozygous allele in most of sub-Saharan Africa. Red blood cells from individuals with this allele are unable to mediate killing of the parasite by platelets (McMorran et al. 2012). Love and colleagues demonstrated that PF4 only accumulated within infected cells and kills parasites by lysing the parasite's digestive vacuole (Love et al. 2012).

The role that platelet-mediated parasite killing has on the outcome following a malarial infection is not known. At least in mouse experiments, it greatly improves survival, but this mechanism of innate immunity is not available to most Africans due to their Duffy negativity status. As most malarial deaths occur in Africa, the extent (if any) that the lack of protection by platelets plays in malaria in Africa has not been studied.

These results show that platelets also have a significant protective role against malaria. This is in contrast with the role that platelets play in the pathology of cerebral malaria. This collection of somewhat disparate effects of platelets have been coalesced into a unified hypothesis by Aggrey et al. (Aggrey et al. 2013) who have suggested that platelets regulate the acute-phase response in a malarial infection. Certainly, platelets produce a large array of immunoreactive molecules, comprising PF4, RANTES, CSCL7 and IL-β1. Acute-phase proteins are the earliest response to infection; are molecules produced by the liver in response to IL-6, IL-1β and TGF-β; and are molecules that are toxic to the parasite and slow the replication of the malarial parasite and promote survival of the host. Later in infection, the parasite mediates an inflammatory reaction and aids in the cytoadhesion of infected red cells which leads to an increase in the pathology of infection and decreases host survival.

However, just to underscore the fact that there is still much to learn about platelets and their role in malaria, Chapman et al. reported data that shows that platelets have the ability to present parasite antigens on a class II MHC molecule (Chapman et al.

2012). They have proteasomes, β_2-microglobulin and HLA, can present parasite antigen and stimulate $CD8^+$ T cells. This raises the important question as to whether platelets also play a role in the adaptive immune response.

Take Home Messages

- Thrombocytopenia is common in malarial infections of all species.
- Platelet bind preferentially to malarial infected red cells.
- Platelet binding only to infected red cells activate and release contents of alpha vesicles.
- Platelets binding to infected red cells results in the death of malarial parasites.
- Platelet Factor 4 (PF4) is the platelet effector molecule from platelets that causes parasite death.
- PF4 binds to the Duffy Antigen. Duffy negative-infected red cells are resistant to platelet-mediated parasite killing.

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Platelets: "First Responders" in Cancer Progression and Metastasis

David G. Menter, Jennifer S. Davis, Stephanie C. Tucker, Ernest Hawk, John D. Crissman, Anil K. Sood, Scott Kopetz, and Kenneth V. Honn

Abstract

A long-standing postulate in oncology is that platelets facilitate cancer metastasis (Menter et al. Cancer Metastasis Rev 33:231–269, 2014; Menter et al. Invasion Metastasis 7:109–128, 1987; Gasic et al. Proc Natl Acad Sci USA 61:46–52, 1968; Woods Bulletin der Schweizerischen Akademie der Medizinischen Wissenschaften 20:92–121, 1964). As their most critical biological response, platelets serve as "first responders" during the wounding process and hemostasis. As a part of the metastatic process, platelet receptors recognize complexes of tumor cell receptors and surface-bound matrix proteins or cellular products as they invade blood vessels. This recognition triggers platelet activation and platelet-tumor cell interactions. Once activated by tumor cells, platelets change shape, degranulate, and release proteins, growth factors, bioactive lipids, and other factors that recruit additional platelets and immune cells along with initiating thrombogenesis. Extensive membrane changes occur at bilayer interfaces between platelets and tumor cells. Tumor cells form extensive membrane/cytoskeletal processes that heavily interdigitate with a central platelet aggregate and involves the uptake of platelet fragments and mitochondria. These interactions are thought to result in the suppression of immune recognition/cytotoxicity or the promotion of cell arrest at the endothelium or entrapment in the microvasculature. These responses all support survival and

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spread of cancer cells and the establishment of secondary lesions. Additional mechanisms of the platelet-metastasis relationship may include the production of platelet exosomes or extravascular migratory behavior of platelets helping to drive cancer progression or preconditioning of secondary metastatic sites. In contrast to the many mechanisms involved in platelet-metastasis relationships, little is known about the role of platelets in precancerous lesion development. This paucity of knowledge exists despite numerous large randomized clinical trials illustrating the cancer preventive effects of nonsteroidal anti-inflammatory drugs (NSAIDs), particularly aspirin in reducing the cancer incidence, mortality, and metastasis. Aspirin covalently acetylates and inactivates platelet cyclooxygenase 1 and thereby eliminates all downstream prostaglandin production from arachidonic acid (AA) by platelets. This includes the key bioactive lipid involved in platelet activation, thromboxane A_2 (TxA_2). Another prostaglandin, prostacyclin (PGI_2), counterbalances and inhibits platelet activation (Honn et al. Science 212:1270–1272, 1981). Metabolically, the genesis of TxA_2 and other bioactive lipids are also impacted by ω-3 polyunsaturated fatty acid substrate substitution for AA. Although not well studied, this places platelets not only at the center of the metastasis discussion but also the progression of premalignancies. Since neoangiogenesis produces leaky blood vessels during early cancer progression, it stands to reason that platelets are the "first responders" to extravasate, activate, and release their stroma-stimulating, proangiogenic, chemoattractive, and immunomodulatory contents. These normal platelet functions and products undoubtedly promote precancerous lesion progression as a series of cyclic amplification events. Platelets are suspected to have a key role within the full spectrum of the cancer progression continuum, which makes limiting their first response an important target for both prevention and therapy.

Platelets in Cancer Metastasis

Platelets as "First Responders"

"First responders" are a perfect moniker for platelets as active participants in the hemostasis, wounding, immune, and metastatic processes (Menter et al. 2014; Gasic et al. 1968; Woods 1964). Platelets are often neglected or overlooked during in vivo experimental or pathologic observations. This is partly due to their small size/volume (mean platelet volumes range is 9.7–12.8 femtoliter or spheres 2.6–2.9 μm in diameter) and the requirement for ultrastructural analysis to effectively observe morphologic or activational structural changes in individual platelets (Fig. 1). Aggregates of activated platelets can be detected by immunohistochemistry at the microscopic level, but this is not routinely done (Qi et al. 2015). As a part of their functional biophysical dynamics, the platelet discoid shape, small size/volume, and physical characteristics cause them to segregate toward the outer fluid shear fields of flowing blood (Fedosov et al. 2014; Kumar and Graham 2012; Tokarev et al. 2011a, b; Lee et al. 2009). Normal human numbers range between 150,000 and 400,000 platelets per microliter (μl), and the concentration of platelets near the vessel wall in vivo is two to three times greater than at the vessel core. Thus, their overall biophysical properties in circulation facilitate platelet distribution toward the endothelial surfaces of vessel walls. Platelet flow patterns, near-wall excess, and proximity enhance their ability to encounter and recognize any lesions in the vascular wall due to a laceration or wound. These platelet recognition properties include the exposure of the subendothelial basement membrane or underlying matrix induced by wounding or endothelial retraction (Menter et al. 1987d; Crissman et al. 1988; Walsh et al. 2015; Kim et al. 2013; Spectre et al. 2012). Platelets can also actively migrate across the inflamed vascular wall in response to stromal cell-derived factor 1 (SDF1 or CXC chemokine ligand 12:CXCL12) and into tumor extravascular spaces (Goubran et al. 2014; Unwith et al. 2015; Schmidt et al. 2012; Kraemer et al. 2010; Brandt et al. 2000; Stone et al. 2012). Platelets can also release SDF1: CXCL12, CXCL1 (GRO-α), CXCL4 (PF4), CXCL5 (ENA-78), CXCL7 (PBP; βTG), and CXCL8 (IL8) among numerous other cytokines and chemokines from storage granules that can potentially initiate the migration and invasion of additional platelets, immune cells (macrophages and leukocytes), endothelial progenitor cells, and tumor cells (Kraemer et al. 2011; Chatterjee et al. 2011; Shenkman et al. 2004; Gleissner et al. 2008). A case for first respondership is further strengthened by the discovery of CXC chemokine receptor 4 and 7 (CXCR4, CXCR7), the cognate receptors for SDF1: CXCL12 on platelets (Rath et al. 2014, 2015; Rafii et al. 2015; Chatterjee et al. 2014b). Expanding upon the concept

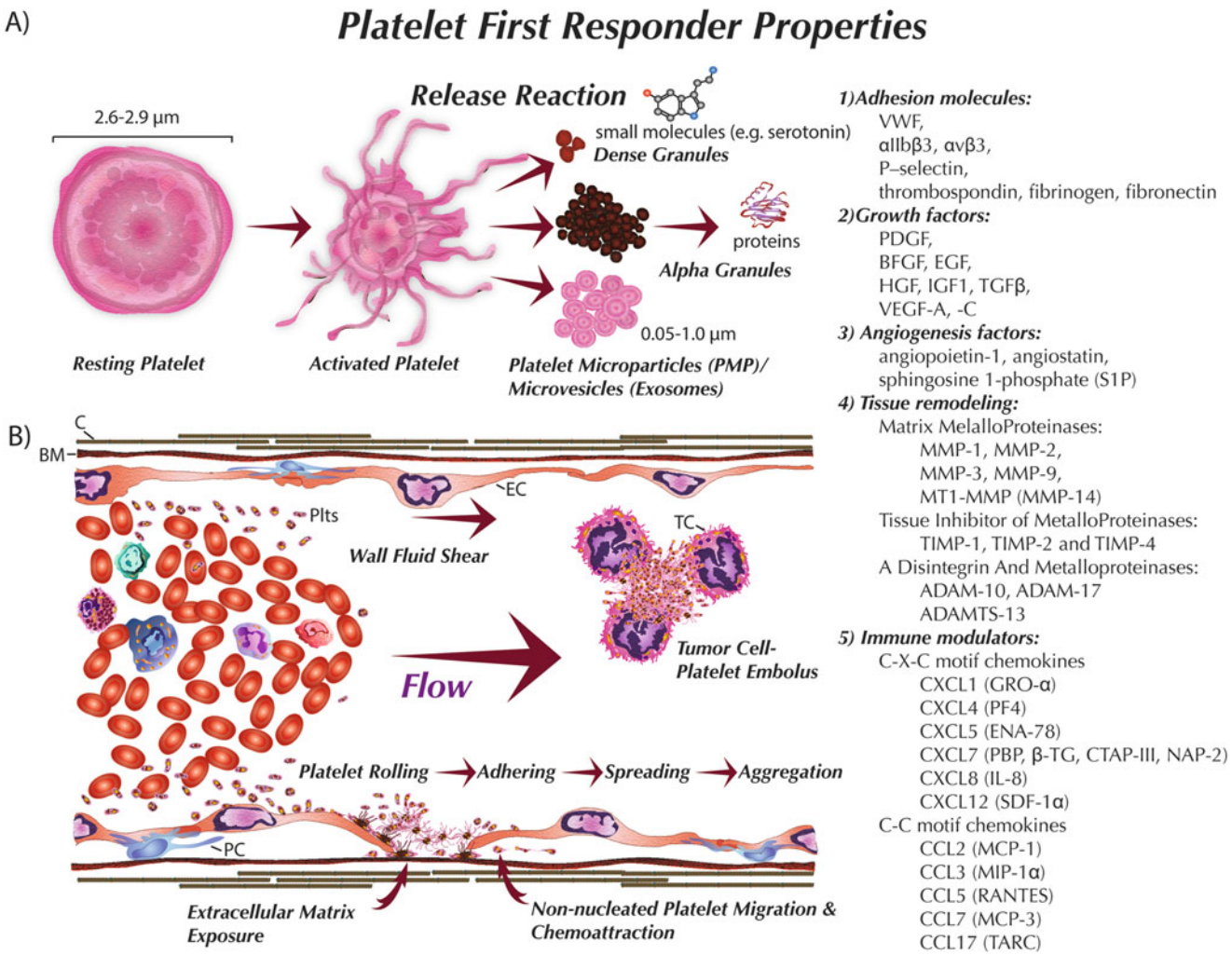

Fig. 1 Platelet first responder properties. (**a**) The activation of platelets is triggered by tumor cells and microenvironmental factors and constitutes an integral part of their first responder properties. This involves shape change and the release reaction. Multiple components that are released into the microenvironment include dense granules, alpha granules, and platelet microparticles (PMP) or microvesicles. The factors released include (1) adhesion molecules, (2) growth factors, (3) angiogenesis factors, (4) tissue remodeling factors, and (5) immune modulators, among others. (**b**) Due to their biophysical and biological properties, platelets accumulate near blood vessel walls within the fluid shear parameters of flowing blood. This enables them to elicit first responses to lesions in the vasculature and the exposure of extracellular matrix. This can lead to the migration of nonnucleated platelets into the perivascular space. As tumor cells enter the blood stream, they can interact with platelets to form heterotypic emboli. *EC* endothelial cell, *PC* pericyte, *BM* basement membrane, *C* collagen, *TC* tumor cell, *PLT* platelet

of migration during potential first responses, platelets are ideally suited for movement because of the highly active cytoskeletal responses linked to activation, adhesion, and aggregation (Menter et al. 1987d, e, 2014; Chopra et al. 1992; Bennett et al. 1999; Jackson et al. 2000; Machlus and Italiano 2013; Qi et al. 2015). Together with an active cytoskeleton, platelets are more streamlined, not only because they are small in size and have minimal displacement volume, but they are also unencumbered by the presence of nuclei, which limits the migration of other immune cells (Breckenridge et al. 2010; Ellingsen et al. 2000; Friedl et al. 2011). The lack of nuclei may also limit the distance that platelets can move into tissue; because of limited protein synthesis capabilities, they cannot indefinitely sustain the replacement of proteins (Bruce and Kerry 1987; Borisova and Markosian 1977). In addition as part of their immune surveillance properties, platelet can also recognize foreign bodies or invading pathogens (Menter et al. 2014; Rossaint and Zarbock 2015; Garraud et al. 2011; von Hundelshausen and Weber 2007). Due to these combined properties, during metastasis, circulating platelets can also elicit a first response to the exposure, sloughing, or active invasion of tumor cells into the blood stream at primary tumor sites. Obstruction of blood flow and angiogenesis associated with primary tumor growth are likely to further enhance the probability of platelet-tumor cell encounters through membrane interactions (Horejsova et al. 1995; Benazzi et al. 2014; Fein and Egeblad 2013). The net result is likely to be platelet activation either to

subdue cells at the primary site or generate tumor cell-platelet emboli in circulation (Menter et al. 1987b, 2014; Crissman et al. 1988). Based on such significant numbers in circulation, small size, biophysical shear properties, adhesion, aggregation, and streamline migration properties, platelets are well suited to serve as "first responders" to a variety of pathologic stimuli, including metastasis.

Tumor Cell: Migration, Invasion, and Intravasation

The proliferation and migration of cancer cells within primary tumors drives a number of events that can impact metastasis (Starke et al. 2014; Gritsenko et al. 2012; Friedl et al. 2012, 2014; Haeger et al. 2014). Direct impact can occur by shedding, sloughing, or active entry of tumor cells into the blood vessels. Based on single cell profiling of circulating tumor cells (CTCs), there is a large diversity of cells found in the circulation that reflect tumor heterogeneity (Deng et al. 2014; Powell et al. 2012). Within the diversity spectrum, CTCs also frequently exhibit stem cell properties (Tang et al. 2015). A variety of triggers can initiate entry of CTCs into the circulation. For example, decreased availability of blood vessels can increase the induction of hypoxia as tumors outgrow their blood supply and release of angiogenesis or wounding related factors (Pasula et al. 2012; Hellberg et al. 2010; Carmeliet 2005). These factors stimulate the formation of new blood vessels that are typically abnormal and leaky, enabling entry of tumor cells into the blood stream (Keskin et al. 2015; Nagy et al. 2012; Fukumura and Jain 2008). Although not extensively studied, there is also potential for leakage or migration of platelets into the tumor that may further enhance the angiogenesis/leaky blood vessel genesis cycle (Kisucka et al. 2006; Goubran et al. 2014; Schumacher et al. 2013; O'Byrne and Steward 2001). More aggressive tumor cells that enter the circulation often undergo epithelial-mesenchymal transition (EMT) (Satelli et al. 2015). In fact, direct signaling between platelets and cancer cells induces an EMT and promotes metastasis in vitro and in vivo (Labelle and Hynes 2012; Labelle et al. 2011; van Es et al. 2014). Cells with EMT characteristics are more fibroblastic in morphology and are typically much more motile and invasive as a result (Labelle et al. 2011; Labelle and Hynes 2012). These EMT cells are prone to actively invade blood vessels by using matrix metalloproteinase (MMP) to digest the extracellular matrix and basement membrane of blood vessels (Nistico et al. 2012). As part of the invasion process, interactions between platelets and tumor cells increase the production of MMP-9 (Labelle et al. 2011). This vascular tumor cell invasive process is termed intravasation and is considered an early dissemination step of the hematogenous metastatic cascade (Fidler 1978).

Tumor Cell-Platelet Recognition/Interaction: A Two-Way Street

Surface receptors abound on both platelets and tumor cells that trigger the primary response mechanisms which drive biologic function (Goubran et al. 2014; Menter et al. 2014). In the case of a nucleated platelets, the lack of nuclei and restricted protein synthesis capabilities limit the adaptation dynamics of platelet surface receptor expression, which is generally established during platelet genesis by megakaryocytes in the bone marrow (Menter et al. 2014). The genesis of heterogeneous populations of platelets that contain carbon copy surface receptor subsets from specific megakaryocytes capable of adapting to disease has not been well established but could have a key impact on cancer if this were true (Bakchoul and Sachs 2015; Penington et al. 1976). In contrast to platelets, nucleated tumor cells have both surface and nuclear receptors that respond to a variety of molecules. Tumor cells also retain the capacity to replace or increase receptor expression by upregulating gene expression and new protein synthesis as they adapt to their microenvironment (Menter et al. 2014).

Integrin and Collagen Receptors

Platelet-tumor cell encounters can be initiated by surface contacts. Integrins are one class of surface receptor molecules that interact with certain extracellular matrix proteins or by direct interactions and consist of α-subunit and β-subunit heterodimer complexes (Fig. 2a) (Menter and Dubois 2012). From the platelet side (pink text), integrin heterodimer receptor complexes include those for vitronectin (α_v/β_3:ITGAV/CD61), fibrinogen (α_{IIb}/β_3:CD41/CD61), laminin (α_2/β_1:CD49b/CD29), and fibronectin (α_5/β_1:ITGA5/CD29) (Menter et al. 2014; Grossi et al. 1987). On the tumor cell side (purple text), many of the same integrin heterodimer complexes can be involved in binding these matrix proteins along with other classes of integrins (Menter and Dubois 2012).

Additional matrix protein receptors include those for collagens. On the platelet side, in this case, collagen receptors are relatively unique to platelets that include GP_{Ib} complexes (consisting of GP_{IX}, $GP_{Ib\alpha}$, $GP_{Ib\beta}$, and GP_V subunits) as well as GP_{VI} (Menter et al. 2014). Tumor cells by contrast can express the entire range of collagen receptors including α_2/β_1 (Menter and Dubois 2012).

Cell Adhesion Molecules, Selectins, and Mucins

Cell adhesion molecules (CAMs) are transmembrane proteins that bind to extracellular matrix and cell-cell adhesion (Fig. 2b). The primary CAM expressed on platelets that

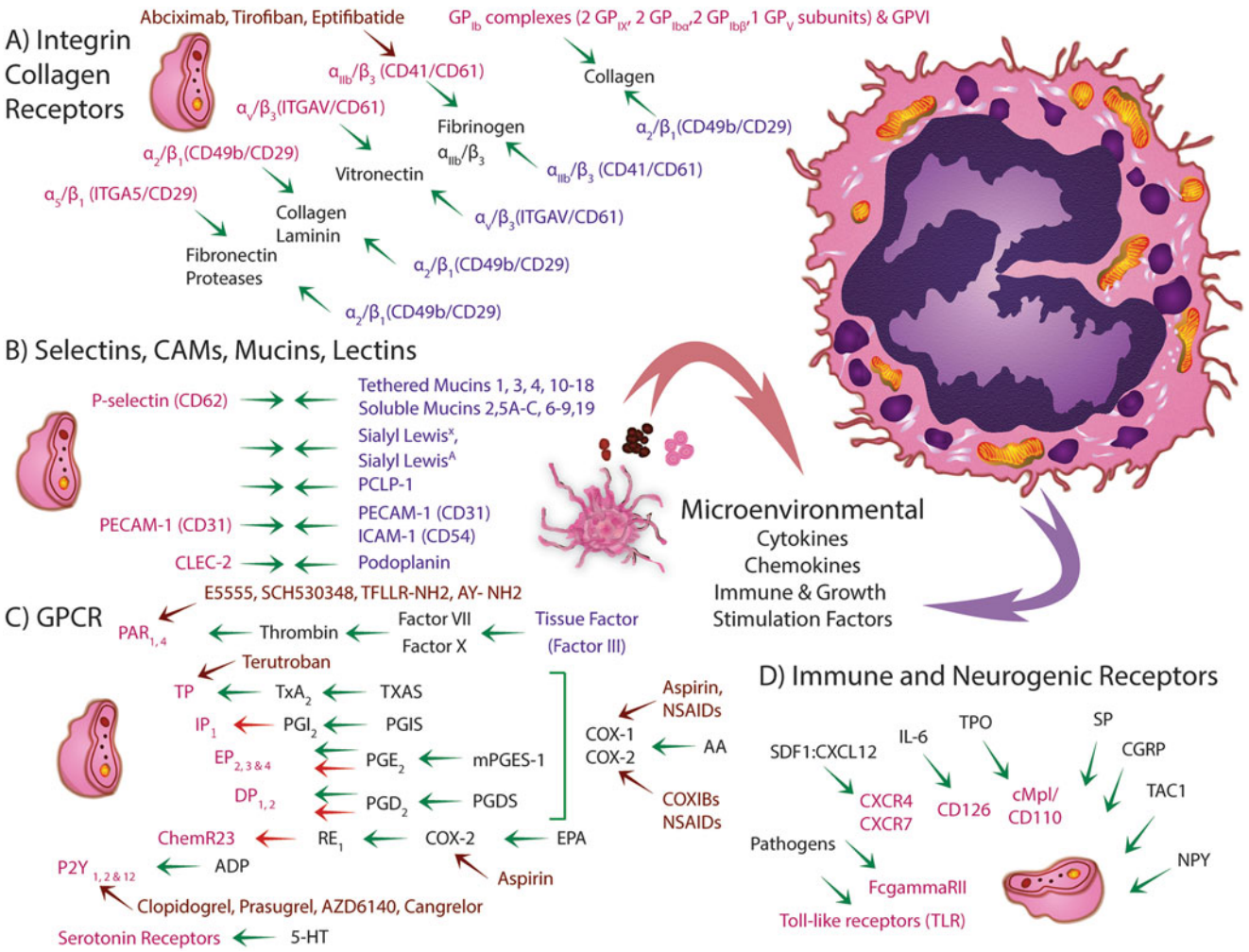

Fig. 2 Receptor and factors that mediate platelet-tumor cell interactions. Integrin and collagen receptors mediate cell-cell interactions between platelets and tumor cells along with collagen and extracellular matrix (ECM) proteins. (**a**) Platelet GPIb complexes recognize vWF tethers, along with thrombospondin, thrombin, $\alpha_M\beta_2$ integrin, kininogen, and clotting factors XI and XII. The GPIb complex consists of two sets of GPIX, GPIbβ, and GPIbα along with a centrally situated GPV protein. Adhesive contacts with collagen exposed by tumor cells are stabilized by GPVI. The $\alpha_{IIb}\beta_3$ integrin has the widest range of interactions and binds to fibrinogen, fibrin, fibronectin, vitronectin, thrombospondin, and vWF. Platelet $\alpha_{IIb}\beta_3$ integrin inhibitors include abciximab, tirofiban, and eptifibatide. Additional platelet and tumor cell integrins include: α_v/β_3(ITGAV/CD61; vitronectin receptors), α_2/β_1(CD49b/CD29; collagen, laminin receptors), and α_5/β_1 (ITGA5/CD29; fibronectin, protease receptors). (**b**) Selectins, cellular adhesion molecules (CAMs), mucins, and lectins can profoundly influence platelet-tumor cell interactions. P-selectin binds to a variety of carbohydrate-rich molecules including: Sialyl Lewis^x or Sialyl Lewis^A as well as tethered (1,3,4,10-18) or soluble (2,5A-C, 6-9,19) mucins and podocalyxin-like protein 1 (PCLP1). Podoplanin on tumor cells can interact with CLEC-2 on platelets. CAMs such as PCAM-1 (CD31) and ICAM-1 (CD54) also regulate platelet tumor cell interactions. (**c**) G protein-coupled receptors (GPCRs) mediate a wide variety of biological interactions. Tissue factor (thromboplastin or factor III) can form complexes with factor VII and factor X to generate thrombin that in turn binds to and activates protease-activated receptors (PAR) 1 or 4 on platelets, which can be inhibited by PAR-specific inhibitors, E5555, and SCH530348. Bioactive lipids also alter platelet-tumor call interactions. Arachidonic acid (AA) is converted by cyclooxygenase 1 and 2 to prostaglandins (PG) and are inhibited by aspirin, NSAIDs, and COXIBs. COX inhibition inhibits multiple downstream steps in the PG cascade. The key target includes the primary platelet-activating pathway: thromboxane A_2 synthase (TXAS) > TxA_2 > TxA_2 receptor (TP) that can be inhibited by a receptor antagonist, terutroban. This activation is countered by the endogenous prostaglandin I_2 synthase (PGIS) > PGI_2 > PGI_2 receptor (IP) inhibition. Other arms of the PG pathway also influence these heterotypic interactions. Another endogenous inhibitor includes resolvin-E1, a product of eicosapentaenoic acid (EPA). Nucleotide receptors can also activate platelets ADP/purinergic G protein-coupled receptors 1 and 12 (P2Y 1, 2 and 12) that are inhibited by clinically relevant receptor antagonists: clopidogrel, prasugrel, AZD6140, and cangrelor. Serotonin (5HT) receptors are also influenced by the uptake and release of 5-HT by platelets. (**d**) Immune receptors are involved in responses to pathogens, including FcgammaRIIB and toll-like receptors (TLR). Platelet genesis is influenced by IL-6 receptors CD126 and thrombopoietin (TPO) receptors cMpl/CD110. Platelet chemotaxis and migration are influenced by stromal cell-derived factor 1 (SDF1 or CXC chemokine ligand 12:CXCL12) through CXCR4 and CXCR7 receptors. Neuropeptides that include substance P (SP), calcitonin gene-related peptide (CGRP), and neurokinin A (NPY) stimulate inflammation when released into the microcirculation, where platelets are key responders that react by modulating arachidonate metabolic pathways. In terms of angiogenic response, the platelet-derived form of the sympathetic neurotransmitter neuropeptide Y (NPY) induces vasoconstriction and ischemic angiogenesis. *Pink text* platelet receptors and factors, *purple text* tumor cell receptors and factors, *maroon text and arrows* inhibitors, *green arrows* stimulation or interaction, *red arrows* inhibition

regulates binding to tumor cells is relatively uniformly expressed (PECAM-1:CD31) (Pang et al. 2015a; Coupland and Parish 2014; Aceto et al. 2014). On the tumor cell side, the expression of surface receptors is not as uniform and can vary extensively with tumor heterogeneity and adaptability (Gunning et al. 2008; Moins-Teisserenc et al. 2015; Raeisossadati et al. 2011). These tumor cell receptors include PCAM-1:CD31, intercellular (ICAM-1:CD54), and epithelial (EpCAM: CD326). Selectins are another family of CAMs consisting of single-chain transmembrane glycoproteins with lectin and cell adhesion properties that bind carbohydrate. Platelets express P-selectin (CD62), which interacts with a variety of carbohydrate-rich molecules produced by tumor cells that include numerically identified mucins that are either tethered (1, 3, 4, 10–18) or free (2, 5A–C, 6–9, 19). Some reports also show evidence of platelets actively recruiting ovarian cancer cells and upregulating tissue factor (Orellana et al. 2015). P-selectin also interacts with Sialyl Lewis^x or Sialyl Lewis^A as well as podocalyxin-like protein 1 (PCLP1) expressed by tumor cells. Platelets can also express C-type lectin receptor 2 (CLEC-2), which interacts with podoplanin on tumor cells (Menter et al. 2014; Goubran et al. 2015).

G Protein-Coupled Receptors (GPCR)

A series of cell-signaling G protein-coupled receptors (GPCRs) can help mediate interactions between platelets and tumor cells (Fig. 2c). Once again on the platelet side, the receptor/signaling networks are very complex (Boyanova et al. 2012; Menter et al. 2014). Tissue factor (thromboplastin or factor III) can form complexes with factor VII and factor X to generate thrombin, which binds to and activates protease-activated receptors (PAR) 1 or 4 on platelets (Menter et al. 2014; Goubran et al. 2014). Tissue factor-dependent thrombin is also enhanced by phospholipid-esterified eicosanoids in agonist-activated platelets (Thomas et al. 2010). The 12-lipoxygenase arachidonate metabolite, 12(S)-HETE, is a bioactive lipid that contributes to endothelial cell retraction as a free acid (Honn et al. 1994). However in its esterified form, after platelet activation, it appears to contribute to coagulation as a result of its translocation to the platelet outer surface. This is supported by the observation that in Scott syndrome, platelets that are defective in cell surface coagulation, the esterified HETE is not presented to the cell surface despite being made to the same degree as in thrombin-stimulated platelets from normal patients. Both the PAR1 and PAR4 agonists (TFLLR-NH_2 and AY-NH_2, respectively) stimulate esterification of 12(S)-HETE with PC and PE to a similar degree (Thomas et al. 2010).

Prostaglandin (PG) receptors are prominent GPCRs expressed by platelets that respond to PGs, which are also important bioactive lipids that profoundly alter platelet function. The PG receptor for thromboxane A_2 (TxA_2), for example, is among the strongest agonists to stimulate platelet activation via thromboxane receptors (TP) (Fontana et al. 2014; Gleim et al. 2013). A differently structured PG is prostaglandin I_2 (prostacyclin) that binds to a separate prostaglandin I_2 receptor (IP_1) and exhibits the strongest inhibition of platelets (Honn et al. 1981; Hubertus et al. 2014). Platelets also express PGE_2 receptors (EP 2, 3 and 4) and PGD_2 receptors that can activate or inhibit platelet function (Petrucci et al. 2011; Cooper and Ahern 1979; Hubertus et al. 2014). Platelets also express additional bioactive lipid receptors. One orphan receptor, *chem*erin *r*eceptor *23* (ChemR23; chemokine-like receptor1: CMKLR1) binds resolvin E_1, a bioactive lipid synthesized from ω3-polyunsaturated fatty acid eicosapentaenoic acid (EPA) found in fish oil that inhibits ADP-induced platelet activation (Fredman et al. 2010; Petrucci et al. 2011). This receptor also serves as a chemokine (C-C motif) receptor-like 2 (CCRL2) that binds chemerin contained in platelets, an adipokine/chemoattractant that has reported roles in inflammation, adipogenesis, and insulin resistance (Du et al. 2009). Tumor cells by contrast express a wide variety of PG receptors depending on tissue origin. Both cyclooxygenase 2 (COX-2) and microsomal PGE_2 synthase1 (mPGES1) are commonly upregulated in cancer, and the production of PGE_2 activates EP receptors (Menter et al. 2010).

Platelets express a number of additional GPCRs. These include thrombin/protease-activated receptors 1 and 4 (PAR 1 and 4) along with ADP/purinergic G protein-coupled receptors 1, 2, and 12 (P2Y 1, 2, and 12) (Franchi and Angiolillo 2015). Both sets of receptors typically trigger platelet activation, and a variety of clinically relevant inhibitors exist for target intervention (Raju et al. 2008). PAR1 and PAR4 are activated following thrombin cleavage that exposes a tethered ligand, which binds to PAR and causes a conformational change and interactions with G proteins (Fu et al. 2015; Wallace and Smyth 2013). Platelet PAR activation can lead to platelet aggregation and thrombosis as well as neutrophil recruitment (Fu et al. 2015; Wallace and Smyth 2013). In contrast, the binding of ADP to P2Y receptors mobilizes intracellular calcium ions via activation of phospholipase C that activates platelets (Cattaneo 2015; Liverani et al. 2014) along with stimulating tumor-cell transendothelial migration and metastasis (Schumacher et al. 2013).

Neurogenic Inflammation and Platelets

The connection between receptor-mediated neurogenic inflammation and cancer is being actively investigated for its role both in potentiating local tumor growth and perineural cancer invasion (Stopczynski et al. 2014; Liebig et al. 2009; Black 2002). Substance P (SP), calcitonin gene-

related peptide (CGRP), and neurokinin A (TAC1) are neuropeptides that stimulate inflammation when released into the microcirculation, where platelets are key responders that react by modulating arachidonate metabolic pathways (Gecse et al. 1999). In terms of angiogenic response, the platelet-derived form of the sympathetic neurotransmitter neuropeptide Y (NPY) is required for capillary angiogenesis during late-phase recovery from ischemic injury (Tilan et al. 2013) and is also produced in animals with hypertension (Ogawa et al. 1992), a condition whose association with cancer has long been suspected (Hamet 1996; Stocks et al. 2012). As a number of cells upregulate NPY to promote metastasis and NPY receptor has been described in the context of its role in cancer, it is conceivable that platelet-derived NPY provided through platelet-tumor cell interactions may also contribute to metastasis (Hong et al. 2015; Medeiros et al. 2012; Magni and Motta 2001).

Serotonin Receptors

Platelet dense granules contain serotonin (5-HT) that is taken up through serotonin transporter (SERT) activity to regulate 5-HT circulating levels (Mauler et al. 2015; Jedlitschky et al. 2012; Linder et al. 2007). More than 95 % of total body 5-HT is synthesized in the enterochromaffin cells of the intestine. Once released from platelets following stimulation, 5-HT binds to cognate receptors and induces vasoconstriction (Mauler et al. 2015; Jedlitschky et al. 2012; Linder et al. 2007). The role of platelet serotonin in malignancy has been pondered for decades (Crawford et al. 1967). Platelet-derived serotonin has been linked to tissue fibrosis (Dees et al. 2011) [considered a form of EMT, reviewed in Tucker and Honn (2013), Crooks et al. (2014), Lopez-Novoa and Nieto (2009)], where platelet aggregation has also been described (Kahaleh et al. 1982). Furthermore, serotonin may be a marker for hepatocellular carcinoma (Pang et al. 2015b; Pai et al. 2009) and has been proposed as a contributing factor to breast cancer (Pai et al. 2009). Platelet serotonergic mechanisms have also been invoked in bladder cancer (Pawlak et al. 2000).

Immune Response Receptors

Additional critical receptors that can impact interactions between platelets and tumor cells include those influencing immunomodulation (Fig. 2d). Platelets express a wide range of receptors that interact with pathogens (Hamzeh-Cognasse et al. 2015) including complement. These receptors include: FcγRII, toll-like receptors (TLR), and also integrins conventionally described in the hemostatic response, such as $\alpha_{IIb}\beta_3$ or GP_{Ib} (Hamzeh-Cognasse et al. 2015). Some of these receptors such as Fcγ receptor IIa can mediate platelet-tumor cell cross talk and initiate tumor cell-induced platelet secretion (TCIPS) (Mitrugno et al. 2014). Interactions with histocompatibility antigen also influence platelet behavior in cancer patients, but this has not been extensively studied (Messerschmidt et al. 1988). Also as previously mentioned, chemokine/cytokine release and subsequent binding to immune receptors on platelets or other cells in the local or systemic environment can stimulate the migration and growth of a variety of cells (Kraemer et al. 2011; Chatterjee et al. 2011, 2014a, b; Shenkman et al. 2004; Gleissner et al. 2008; Rath et al. 2014, 2015; Rafii et al. 2015).

Cancer-Induced Thrombocytosis

Finally, cancer-induced thrombocytosis significantly increases the number of circulating platelets and is linked to tumor cell proliferation (Bouvenot et al. 1977; Honn et al. 1992; Levin and Conley 1964; Cho et al. 2012). This constitutes an important link to Trousseau's syndrome, but the mechanisms responsible remained poorly understood until recently (Stone et al. 2012) when a multicenter study involving 619 ovarian cancer patients examined the associations between platelet counts and disease outcome (Stone et al. 2012; Davis et al. 2014). During this study, thrombocytosis was elevated in association with circulating levels of thrombopoietin (TPO) and interleukin-6 (IL-6). This elevation was associated with advanced disease and shortened survival in ovarian cancer patients and was verified in orthotopic mouse models (Stone et al. 2012). In pancreatic cancer studies as well, paraneoplastic thrombocytosis predicts poor prognosis in patients with locally advanced disease (Chadha et al. 2015). These tumor-liver-bone marrow-platelet feedback mechanisms are thought to occur through IL-6 receptors: CD126 (Marino et al. 2013) and TPO receptors: cMpl/CD110 (Hitchcock and Kaushansky 2014).

Anticoagulant Therapy for VTE Prevention in Cancer Patients

It is estimated that individuals with cancer have a fivefold higher incidence of venous thromboembolic events (VTEs) than the general population (Chao et al. 2011). Chemotherapy can also contribute to elevated levels of thrombosis. In healthy individuals, venous thrombosis can be the first manifestation of malignancy. Since the 1970s when the Veterans Administration Cooperative Study Program first tested warfarin in cancer patients, many other studies have examined agents that interrupt the coagulation pathway as a means to attenuate VTEs and impede cancer progression (Chao et al. 2011). Unfortunately, the doses of warfarin required to achieve therapeutic results were not without side effects

(Zacharski et al. 2005), and heparin required continuous intravenous administration (Hull et al. 1986). As a result of numerous clinical trials, low molecular weight heparin (LMWH or nadroparin) replaced unfractionated heparin (UFH) nearly 20 years ago as the preferred treatment and prophylaxis agent for several reasons (Chao et al. 2011; Salzman et al. 1980). Its chemical structural properties allow it to specifically inhibit factor Xa without all of the off-target serine protease inhibition seen with UFH. Compared to UFH, LMWH is easier to administer and significantly lowers recurrent thrombosis, major bleeding, and mortality (van Dongen et al. 2004). There is some suggestion that the beneficial effects of LMWH in treating cancer patients may be direct against the tumor cells in addition to the anticoagulant effect (Barni et al. 2014). Even with these advances and new reagents, a major concern to consider is the potential to induce heparin-induced thrombocytopenia (Wharin and Tagalakis 2014; Greinacher 2015).

To Decorate, Cloak, or Hold Tumor Cells: That Is the Question

As part of their function in immune surveillance, platelets interact with viral particles, bacteria, and parasites as well as virally transformed tumor cells (Chabert et al. 2015; Hamzeh-Cognasse et al. 2015; Kofman et al. 2013; Ahmed et al. 2014). Many of these interactions essentially help to decorate pathogens and trigger platelet aggregation and the release of factors that stimulate leukocyte activation and neutrophil extracellular trap (NET) formation (Hamzeh-Cognasse et al. 2015). In contrast, tumor cell-platelet interactions are postulated to suppress immune recognition/cytotoxicity and promote arrest at the endothelium or entrapment in the microvasculature. These responses support survival and spread of cancer cells and the establishment of secondary lesions. The current dogma suggests that once tumor cells enter the circulation, they can become enveloped or decorated by a coating of platelets that shields them from recognition by cytotoxic immune cells. While this notion has received support from numerous investigators, it may not be exactly what occurs. When examining heterotypic aggregates that formed during tumor cell-induced platelet aggregation (TCIPA), the platelets tend to cluster at the center, not the external surfaces (Menter et al. 1987a, c) (Fig. 3). In most cases the tumor cells are not completely enveloped enough following TCIPA to fully encase and hide the tumor cells from immune cells (Menter et al. 1987a, c, e). Furthermore, from a logistical standpoint, the feasibility of completely covering the large tumor cell surface area with much smaller platelets is in doubt.

Platelet Changes in Cancer Patients

Recent studies have demonstrated that there are profound ultrastructural changes in platelets from cancer patients as compared to normal controls or individuals with benign disease. Electron cryotomography (cryo-ET) was used to examine platelets from patients with invasive ovarian cancer and controls (either benign adnexal mass or free from disease). Significant morphological differences were detected between the cancer and control platelets including disruption of microtubules as well as altered numbers of mitochondria (Wang et al. 2015). Furthermore, RNA-seq studies have revealed that molecular pathway changes in tumor-educated blood platelets (TEPs), thus connecting them to systemic and local responses to tumor growth resulting in altered RNA profiles (Best et al. 2015).

Heterotypic Emboli, Platelet Interactions, and Extensive Membrane/Structural Changes

The heterotypic contacts between platelets and tumor cell lead to dynamic focal interactions during TCIPA (Menter et al. 1987a, c, e). As platelets cluster centrally during heterotypic aggregate formation, they change shape and degranulate and release proteins, growth factors, bioactive lipids, and other factors that recruit additional platelets toward the central core of the interaction (Menter et al. 1987a, c, e). Extensive membrane changes occur at the membrane interfaces between platelets and tumor cells. There is very little data on whether the tumor cells or platelets drive these membrane interactions, but they appear to be bidirectional. Extensive membrane/cytoskeletal tumor cell processes heavily interdigitate with a central platelet aggregate, demonstrating a very active process. As these interactions progress, extensive uptake of platelet fragments and mitochondria occurs into the tumor cells in vitro (Menter et al. 1987a, c, e). This interdigitation and uptake of platelet fragments by tumor cells are also observed in vivo following tail vein injections of syngeneic tumor cells into mice (Crissman et al. 1985, 1988; Menter et al. 1987b).

The engulfment of platelets or platelet particles such as mitochondria may be a form of emperipolesis or entosis. These are processes whereby cells take up other cells without degradation with varied outcomes (Xia et al. 2008; Rastogi et al. 2014). This is reminiscent of frustrated phagocytosis where organisms ride along the plasma membrane or become engulfed only to be subsequently released. Some metastatic tumor cells even take on behaviors of M2 polarized macrophages (Caruso et al. 2012; Djaldetti and Strauss 1982).

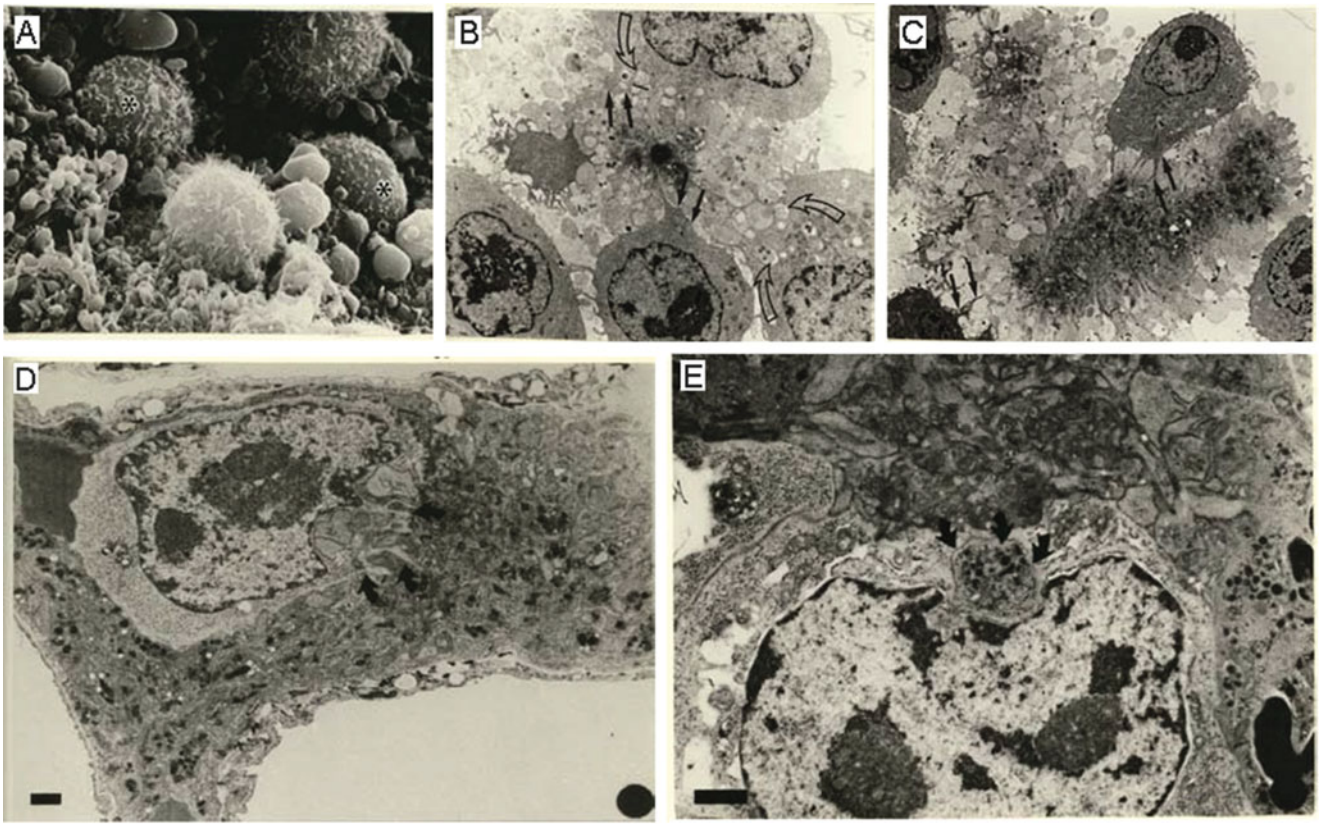

Fig. 3 Early events in platelet-tumor cell interactions. (**a**) Elutriated W256 tumor cells are shown interacting with platelets in an aggregometer cuvette at the plateau phase (10 min). A reduction in tumor cell surface microvilli occurs by this time (*asterisks*, scanning electron micrograph; magnification x3900). (**b**) Elutriated W256 tumor cells project small processes (*arrows*) into platelet aggregates by midphase of aggregation that become more (**c**) prominent and protrude deeper into forming platelet emboli as these interactions plateau (*arrows*) that associates with tumor cell engulfment of platelet fragments (*open arrows*) (transmission electron micrographs; magnifications—(**b**) x3000, (**c**) x4300). (**d**) Elutriated Lewis lung tumor cells form intravascular aggregates in the mouse lung that involves process penetration (*arrows*) into syngeneic C57Bl/6 platelet emboli, which occurs 2 min after tail vein injection and (**e**) engulfing platelet fragments (*arrows*; transmission electron micrographs; magnifications—(**d**) x6800, (**e**) x12,000, *scale bars* 1 μm). (**a–c**) Reprinted from Honn et al. (1983) with permission from S. Karger AG, Basel; (**d, e**) courtesy of Menter

While genomic and epigenetic elements are fundamental to the cancer continuum, the contribution of exosomes/microvesicles, free nucleic acid, and cytoplasmic elements is just as intriguing. It has been suggested that the reaction of the mitochondrial genetic system to genomic (nuclear DNA) stress response genes leads to mitochondrial dysfunction and could account for the marked increase in metabolic diseases and cancer (Wallace 2005; Brahimi-Horn et al. 2015). Studies have demonstrated that tumorigenic phenotypes can be down-modulated when mitochondrial DNA is ablated and can be complemented by reintroduction of mitochondria (Cavalli et al. 1997). Therefore, hyperpolarization of mitochondria in activated platelets and the observation that platelets release mitochondria to potentiate immune response (Zharikov and Shiva 2013; Boudreau et al. 2014) raise the specter that platelet mitochondria potentially make a direct contribution to malignancy by regulating tumor cells. Whether platelet mitochondria compensate for tumor cell mitochondrial deficits or augment some tumor cell function through genetic cross programming remains to be determined. With respect to the latter idea, mitochondrial transcription factor binding sites are seen throughout the nuclear genome. Coordinated regulation of nuclear genes in response to mitochondrial stress or mitochondrial uptake has not yet been established, but it is a topic being scrutinized (Haynes et al. 2013).

Secondary Sites, Exosomes, and Extravasation: Differences or Similarities?

Tumor cell exosomes play key roles in cancer progression and metastasis (Melo et al. 2014, 2015; Kahlert and Kalluri 2013; Milane et al. 2015; An et al. 2015; Robbins and Morelli 2014; Colombo et al. 2014). Functionally, these exosomes can influence the biology of both the local tumoral

and systemic environments (Kahlert and Kalluri 2013). Tumor cell exosome biogenesis often begins with endocytosis, then endosome genesis, followed by the formation of multivesicular bodies (MVBs) or by microvesicle formation (Huber and Holvoet 2015; Zhang et al. 2015). Exosomes formed within MVBs are released following membrane fusion into the surrounding microenvironment or circulation. Locally, release of tumor cell exosomes into the microenvironment can influence proliferation and chemoresistance and initiate fibroblast or immune cell activation and recruitment along with stimulating angiogenesis. Systemically, tumor cell exosomes can stimulate a hypercoagulable state, initiate preneoplastic niches at secondary metastatic sites, recruit bone marrow-derived hematopoietic and immune cells, or elicit immunosuppression (Lima et al. 2013). Classically by contrast, platelets contain numerous electron-dense subcellular organelles called alpha granules, electron-dense and very electron-dense granules, lysosomes, and dense bodies (Fukami and Salganicoff 1977). The many factors contained in these granules constitute what is commonly found in serum used to grow cancer cells in tissue culture and in regenerative medicine (Dhillon et al. 2014; Burnouf et al. 2013; Ross et al. 1978). These organelles are sometimes called secretory granules, since the appropriate stimuli causes degranulation that deposits granule contents in the extracellular surroundings. These organelles were identified and visualized by electron microscopy, microprobe analysis, secretion experiments, and subcellular fractionation studies from patients with platelet storage pool deficiency diseases. In many respects, this degranulation process can be equated with the formation of platelet microparticles (PMP), which are essentially very exosome-like (Goubran et al. 2015; Mezouar et al. 2014). Platelet extravascular migratory behavior and exosome deposition is likely to drive cancer progression or preconditioning of secondary metastatic sites (Goubran et al. 2015). Preventing platelet activation inhibits the P-selectin and integrin-dependent accumulation of cancer cell microparticles and reduces tumor growth and metastasis in vivo (Mezouar et al. 2015).

Blood-borne CTCs are commonly found in the blood stream of patients with metastases (Aceto et al. 2014; Li et al. 2015; Joosse et al. 2015). In a patient study of CTCs isolated from breast and prostate cancer patients, their presence correlated with poor prognosis (Aceto et al. 2014). This study focused on comparing single CTCs to CTC clusters and found that markers for platelets were present in both populations (Aceto et al. 2014). The presence of these clusters was also associated with high plakoglobin expression and poor prognosis (Aceto et al. 2014). Since plakoglobin plays an important role in the regulation of CAM interactions (Ilan et al. 2001; Rival et al. 1996; Reynolds et al. 1992, 1994), its upregulation may influence platelet/tumor cell heterotypic CTC cluster formation. In a separate randomized phase II trial, therapeutically targeting platelet function did not significantly change the number of CTCs in circulation in patients with metastatic breast cancer. Unfortunately, the number of circulating cells was lower than expected, so a more definitive study is needed (Roop et al. 2013). In early mouse studies, although large numbers of CTCs may be present in circulation, only those with specific characteristics survive to establish metastasis (Fidler and Nicolson 1978; Fidler 1975; Hart and Fidler 1980). In other mouse hematogenous metastasis studies, cell arrest at secondary sites precedes the establishment of lesions. This may occur by a variety of mechanisms. Tumor cell interactions directly with the vascular endothelium can initiate the process. In this case, the engagement of platelets can occur during or after tumor cell arrest has occurred, and platelets can fill in around arrested tumor cells. Tumor cell-platelet heterotypic interactions can also occur in suspension in flowing blood to initiate TCIPA. The resulting heterotypic platelet-tumor cell emboli can then undergo attachment or entrapment in secondary organ sites. Platelet-tumor cell emboli initially interact with vascular surfaces at cellular junctions or gaps in the endothelial cell monolayer (Menter et al. 1987e). Platelets are also capable of forming chains that tether tumor cells to these attachment sites (Menter et al. 1987e). As these cellular interactions progress, platelets engage both endothelial cell surfaces and gaps in the endothelial cell monolayer facilitating the linkage to tumor cell membranes (Menter et al. 1987e). In other studies, agent-based, computational, theoretical modeling simulated the basic dynamics of circulating tumor cell adhesion involving endothelial, neutrophil, and platelet interactions and proposed multiple therapeutic targets involving platelets (Uppal et al. 2014). Other studies have utilized intravital microscopy to visualize human red fluorescent protein (RFP) expressing HT29 (RFP-HT29) colon cancer cells injected into the spleens of GFP expressing nude mice (Tanaka et al. 2014). These studies revealed extensive interactions between GFP platelets and RFP-HT29 in liver sinusoids (Tanaka et al. 2012, 2014). Intravital imaging has also been done using a syngeneic C57/Bl6 mouse model, and interactions were shown to involve $\alpha_v\beta_3$ integrins on B16 melanoma cells to $\alpha_{IIb}\beta_3$ on platelets using blocking antibodies (Lonsdorf et al. 2012).

Tumor cells can exit the vasculature at secondary sites (Crissman et al. 1985), and platelets can facilitate this process known as extravasation (Coupland et al. 2012; Crissman et al. 1985; Fein and Egeblad 2013; Pang et al. 2015a; Schumacher et al. 2013). Thrombin activation involving platelet membranes can facilitate the activation of ECM degradation by tumor cells in a P-selectin and

$\alpha_{IIb}\beta_3$-dependent manner (Pang et al. 2015a). Taken together these studies support the notion of platelet involvement in the establishment of hematogenous metastasis.

New Thoughts: Platelets in Premalignancy

So What About Aspirin, Arachidonic Acid, and Omega-3 PUFA?

Meta-analyses of large randomized clinical trials (Bousser et al. 2011; Chan et al. 2012; Chan and Cook 2012; Cuzick et al. 2015; Goldstein and Rothwell 2012; Langley and Rothwell 2013, 2014; Rothwell 2013a) show that aspirin reduces the incidence (Algra and Rothwell 2012), metastasis (Rothwell et al. 2012), and mortality (Rothwell et al. 2012) of gastrointestinal and other cancers, especially adenocarcinomas (Algra and Rothwell 2012; Chan et al. 2012; Cuzick et al. 2015; Langley and Rothwell 2013; Rothwell 2013a, b; Rothwell et al. 2012). Despite substantial cumulative knowledge regarding cancer prevention by aspirin (Umar et al. 2016), little is known about the role of the primary target found in platelets during precancerous lesion development. Aspirin covalently acetylates Ser-530 and irreversibly inactivates platelet cyclooxygenase 1 (DeWitt et al. 1990; Rowlinson et al. 2000), thereby eliminating all downstream prostaglandin production from arachidonic acid by platelets. This includes the key bioactive lipid involved in platelet activation, thromboxane A_2 (TxA_2) (Fontana et al. 2014; Fitzpatrick and Gorman 1977; Hammarstrom and Falardeau 1977). Pronounced elevation of circulating TxA_2 levels accompanies CRC progression in familial adenomatous polyposis (FAP) patients (Li et al. 2015). As a strong platelet TP receptor agonist, TxA_2 produced by TxA_2 synthase in platelets is counterbalanced by an equally strong platelet inhibitory response by IP_1 receptors. IP_1 receptors are activated by PGI_2 that is produced by PGI_2 synthase in endothelial cells lining the vasculature. Both TxA_2 and PGI_2 have epoxide bonds that contain considerable molecular strain, which limits their half-life in circulation. Activation by TCIPA shifts this delicate eicosanoid balance triggering multiple pro-carcinogenic responses centered on platelets.

Diets rich in ω-3 PUFA, EPA, and docosahexaenoic acid (DHA) inhibit carcinogenesis (Holla et al. 2008; Schroeder et al. 2007; Yang et al. 2006, 2014a, b). EPA feeds into the COX-1 and COX-2 pathways to produce TXA_3 and PGE_3 lipid metabolites that are ineffective at PG receptor activation and protect against CVD and lung or colon tumorigenesis (Algra and Rothwell 2012; Chan et al. 2012; Chan and Cook 2012; Cuzick et al. 2015; Goldstein and Rothwell 2012; Holla et al. 2008; Langley and Rothwell 2013, 2014; Liyasova et al. 2010; Macdonald et al. 1999; Pinckard et al. 1968; Rothwell 2013a; Rothwell et al. 2012; Schroeder et al. 2007; Yang et al. 2006, 2014a). Taking aspirin in combination with EPA and DHA leads to the formation of resolvins, maresins, and protectins (Serhan et al. 2006, 2009; Spite et al. 2014; Spite and Serhan 2010; Krishnamoorthy et al. 2015; Serhan 2014). Resolvins were originally named for their ability to resolve inflammation. More specifically, aspirin-inactivated COX-2 generates a longer half-life R-epimer of resolvin-D_1 (Arita et al. 2005; Serhan et al. 2004; Sun et al. 2007) from DHA and resolvin-E_1 from EPA (Dona et al. 2008; Pirman et al. 2013). Although not well studied, this places platelets not only at the center of the metastasis discussion but also the progression of premalignancies.

Leaky Vessels, Extravasation, and "First Response"

The role of angiogenesis in premalignancy remains an important question, particularly how early this change occurs (Menakuru et al. 2008) (Fig. 4). Biomarkers of this process include factor VIII, CD31, and CD34. Increased microvascular density can occur in ductal carcinoma in situ (DCIS) (Menakuru et al. 2008; Sapino et al. 2001). These premalignant lesions range in size from 1 to 1.5 cm (Allegra et al. 2009). Newly formed vessels can be identified by CD105 (Fox and Harris 2004; Smith et al. 2012; Ratajczak et al. 2012; Dubinski et al. 2012; Marioni et al. 2011; Sapino et al. 2001). Platelet-derived thrombospondin 1 helps regulate angiogenesis and serves as a potential biomarker (Zaslavsky et al. 2010). The presence of highly permeable, disorganized, poorly formed, and leaky blood vessels contribute to progression in advanced cancers (Fang et al. 2011). These leaky vessels provide ample opportunity for platelet first responders to extravasate and enter the precancerous microenvironment. Once in the perivascular space, platelet activation triggers the release of growth-promoting factors found in serum. These serum factors are highly likely to provide a permissive and growth-promoting microenvironment for transformation.

Angiogenic and immune stimulation often switch on during premalignancy, before cells achieve the invasive state (Ferrati et al. 2012; Folkman et al. 1989). Many factors influence this switching, including microRNAs (Bergers and Benjamin 2003; Grandis and Argiris 2009). Switching has been elegantly demonstrated in the adenoma-adenocarcinoma transition of colorectal cancer and the transition from DCIS to invasive ductal carcinoma of the breast. Specifically, the initiation of angiogenesis accompanies dysplasia during adenoma formation in colorectal cancer progression (Staton et al. 2007) and hyperplasia during DCIS

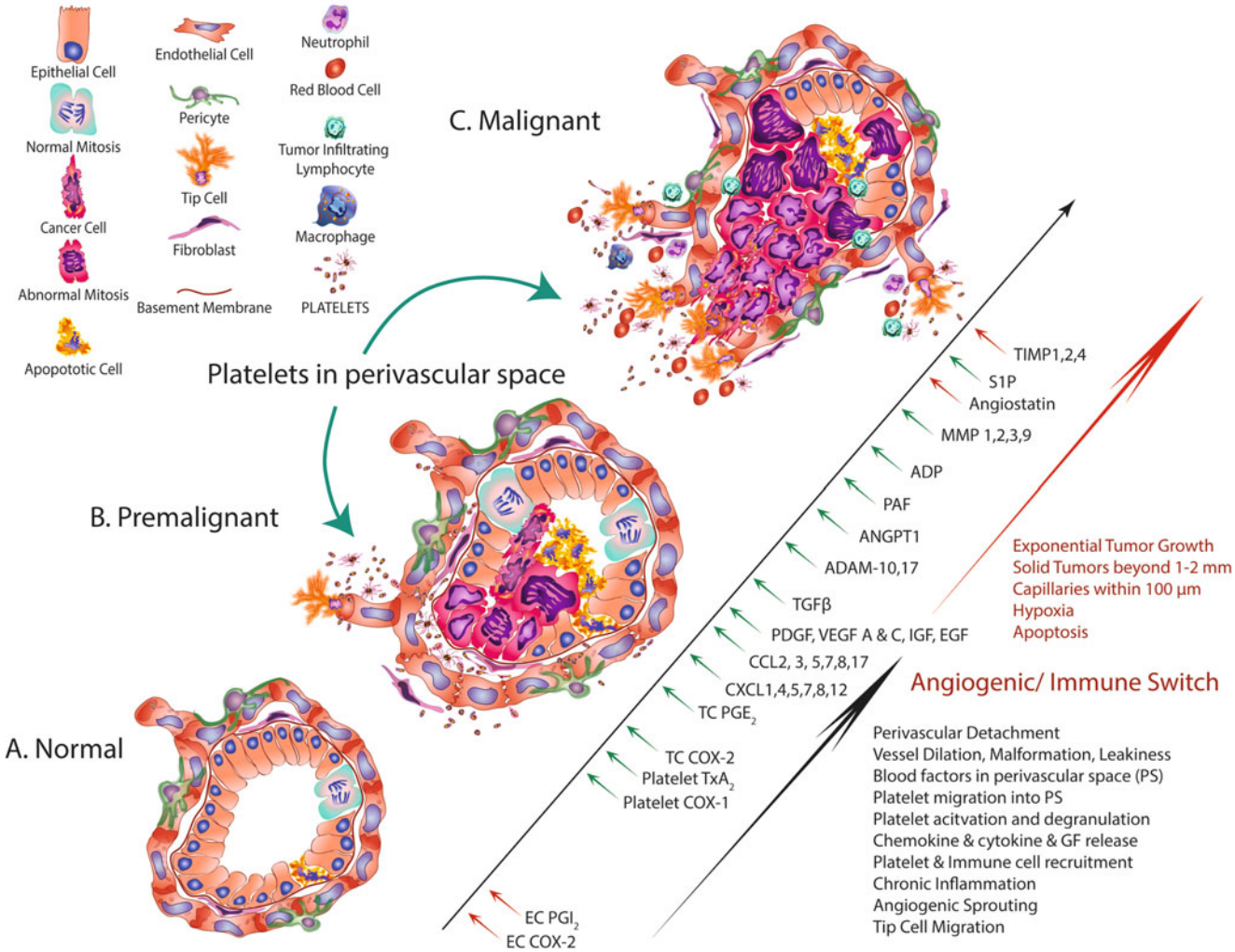

Fig. 4 Platelet influences on angiogenic and immune switches. Angiogenic and immune switches can turn on at different stages of carcinogenesis. (**a**) Biological maintenance of normal tissue involves a delicate balance between cell growth and apoptosis and homeostasis that is regulated by immune surveillance, tissue repair, and immune clearance. (**b**) Premalignant disease may involve the earliest stages of angiogenic and inflammatory switch activation. Premalignancy-associated inflammation triggers immune cell infiltration in response to cytokines and prostaglandins that promotes vessel dilation and the detachment of pericytes from blood vessels. During these early stages of premalignancy, blood factors accumulate in the perivascular space. This provides the opportunity for platelets to migrate into the perivascular space along with activation and the release of platelet granules containing vascular endothelial cell growth factor (VGEF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epithelial growth factor (EGF), and angiopoietin-2 (Ang2) that enhances both angiogenic and immune switch activation. (**c**) The onset of malignancy, heightened hypoxia, and apoptosis further increases vessel leakiness, platelet activation, platelet migration, and granule release. The cycle accelerates as angiogenic sprouting and tip cell-mediated migration into the growing tumor lead to further recruitment of additional platelets, immune cells, and tumor cell migration and proliferation as cancer progression continues

progression to invasive breast cancer (Bluff et al. 2009). Mechanistically, mice that lack histidine-rich glycoprotein (HRG) exhibit an accelerated angiogenic switch and enhanced platelet activation, inducing molecular changes in the pre-tumorigenic environment accompanied by increased survival, angiogenesis, and EMT (Cedervall et al. 2013). Although more studies are needed, these effects suggest an early, vital role for platelets early in the premalignancy to malignancy transition. Abnormal or leaky blood vessels that arise during tumor-initiated angiogenesis provide adequate opportunity for the leakage or migration of platelets into the precancerous microenvironment. Subsequent activation and release of their stroma-stimulating, proangiogenic, chemoattractive, and immunomodulatory contents can then promote precancerous lesion progression as a series of cyclic amplification events. In support of this hypothesis, release of VEGF and other platelet factors occurs during early breast cancer (McDowell et al.

2005), and platelet $GP_{Ib\alpha}$ also increases in invasive intraductal breast carcinoma tissues (Oleksowicz et al. 1997). These early angiogenic and immunogenic switching events during premalignancy suggest that platelets are likely contributors to early cancer progression along with potential targets for early diagnosis (Han et al. 2014).

Immunotherapy?

Platelets are derived from megakaryocytes (Menter et al. 2014; Machlus and Italiano 2013), arising during the process of megakaryopoiesis from hematopoietic stem cell lineage that share commonalities with both lymphoid and granulocytic cells prior to differentiating toward hematopoietic progenitors. Specifically, in the bone marrow, hematopoiesis produces hemangioblasts that give rise to *hematopoietic stem* cells (HSCs) and *multipotent progenitor* cells (MPP) (Deutsch and Tomer 2013; Meng et al. 2012; Thon and Italiano 2010; Akbar et al. 2011). Genesis from MPP forms *common myeloid progenitor* cells (CMP) (Deutsch and Tomer 2013; Akbar et al. 2011; Thon and Italiano 2010; Meng et al. 2012) that give rise to *megakaryocyte-erythroid progenitor* (MEP) cells followed by *megakaryopoietic progenitors* (MKP) (Kanz et al. 1987). MKP then generate immature—followed by mature—megakaryocytes before finally generating platelets (Deutsch and Tomer 2013; Geddis 2010; Machlus and Italiano 2013; Tijssen and Ghevaert 2013; Meng et al. 2012). Immune thrombocytopenia (ITP) involves cellular checkpoints during the normal regulation of immunological self-reactivity and the development of B and T cells (Cines et al. 2009). This may occur through cell deletion, gene editing, induction of anergy, and extrinsic cellular suppression. When immune checkpoints fail, tolerance to self-antigens may be downregulated (Toltl et al. 2011; Cines et al. 2009). Checkpoint failure is a key target of immune checkpoint blockers that are being used in combination with interferon to overcome immune tolerance (Rafique et al. 2015). Importantly, platelet genesis and lineage development are also influenced by interferon (Rivadeneyra et al. 2015; D'Atri et al. 2015). With the advent of immune checkpoint cancer therapy, the influence of these agents may cross over to impact platelet biology and function (Topalian et al. 2015; Miller and Sadelain 2015; Gubin et al. 2014), which may also influence immune-related adverse events that are autoinflammatory in nature (Weber et al. 2015). Most notably, preclinical data demonstrate that pharmacologic inhibition or genetic deletion of COX-1 and COX-2 synergizes with anti-PD-1 blockade to induce the eradication of tumors more effectively than PD-1 treatment alone (Zelenay et al. 2015). Aspirin treatment was highly effective in synergizing with anti-PD1, but platelet function was not followed in these studies and deserves additional study (Zelenay et al. 2015). In view of their "first responder" properties, it is critical to develop a better understanding of this role or potentially harness platelets in some fashion to alter checkpoint blockade. One could envision taking bone marrow aspirates from cancer patients and engineering in vitro cultures of megakaryocytes (Nishikii et al. 2015; Hatami et al. 2015; Meinders et al. 2015; Panuganti et al. 2013; Thiele et al. 2012; Emond et al. 2012; Pallotta et al. 2009) to alter tumor recognition and inflammatory responses and reinfuse these reengineered platelets though adoptive bone marrow transfer.

Platelets: "First Responders" in the Complete Cancer Continuum

Platelets as vital contributors to the early stages of cancer formation are a new concept. Support for this notion comes from a variety of sources. One line of evidence is the long-standing link between chronic inflammation and the risk of developing colon cancer. Patients with inflammatory bowel disease or Crohn's disease, for example, are at high risk for developing colon cancer (Herszenyi et al. 2015; Burisch and Munkholm 2015; Sanduleanu and Rutter 2014). A potential association with platelet biology may be found in mean platelet volume changes that accompany Crohn's inflammatory disease states, among others (Kilincalp et al. 2015; Tang et al. 2015; Huang et al. 2013; Harris et al. 2011). Similarly, changes in mean platelet volume are found during adenoma formation (Kilincalp et al. 2015) along with colorectal cancer (Li et al. 2014; Kemal et al. 2014).

Additional support for this notion comes from $Apc^{min\backslash+}$ mouse crossbreeding with $P\text{-}selectin^{-\backslash-}$ (CD62P) knockout mice, which lack expression on activated platelets and reduced VEGF expression (Qi et al. 2015). These crosses resulted in reduced formation of low- and high-grade adenomas accompanied by decreased vessel density (Qi et al. 2015). This study also examined human adenomas and stages I–IV of CRC progression by IHC for CD41 (integrin GP_{IIb} subunit) positivity. Similar levels of platelet staining were observed in the polyp and stage I CRC tissues (Qi et al. 2015), suggesting that the platelets were already involved at the polyp stage. This CD41 staining increased in intensity with each progressive stage II–IV of CRC tumor development. Our group has also recently observed extravascular platelet structures in $Apc^{min\backslash+}$ polyps (Fig. 5).

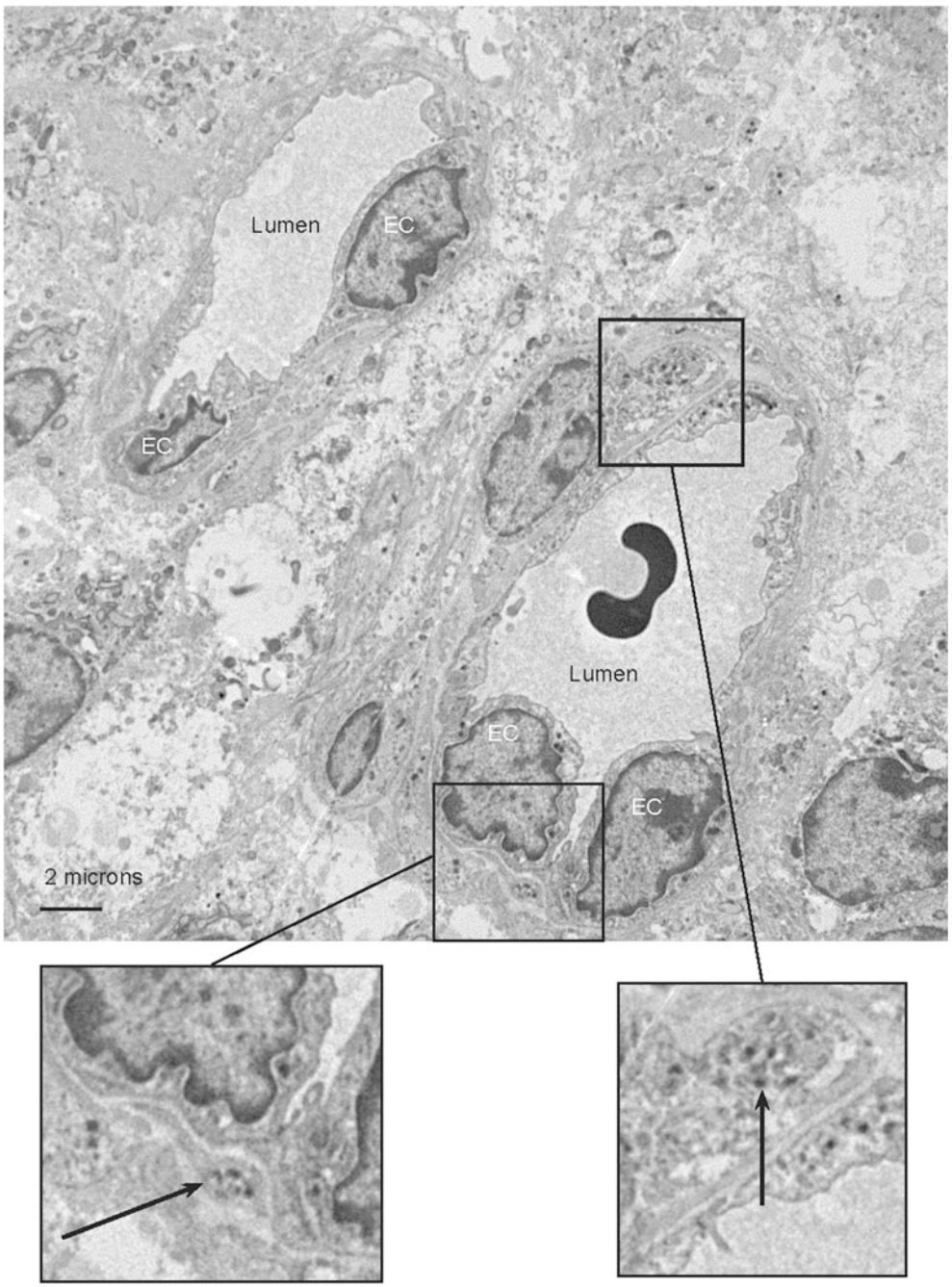

Fig. 5 Platelet migration into adenoma perivascular spaces. Ten-week-old adenomatous polyposis coli $Apc^{min\backslash+}$ mice were euthanized and necropsied, and the intestinal tract was excised and fixed. Adenoma polyps were dissected and processed for transmission electron microscopy. The examination of polyp tissue revealed platelet structures in the perivascular space of blood vessels (*arrows*; *insets*). These platelets were underlying endothelial cells (EC) and had exited the vascular lumen (x4000, *scale bars* 2 μm)

These data, along with our previous studies illustrating the dynamic activation of platelets in heterotypic platelet-tumor cell aggregates and syngeneic intravascular metastasis, highlight a role for platelets throughout the cancer continuum. Collectively, these data strongly suggest that altering the "first responder" capabilities of platelets is an important target for cancer prevention and therapy.

Take Home Messages

- Platelets are "First Responders" as active participants in the hemostasis, wounding, immune, and metastatic processes.
- Platelets are often neglected or overlooked during in vivo experimental or pathologic observations due to their small size and lack of defining nuclei.
- Surface receptors abound on both platelets and tumor cells trigger the primary response mechanisms that drive biologic function.
- When examining heterotypic aggregates that form during tumor cell-induced platelet aggregation (TCIPA), the platelets tend to cluster at the center.
- Platelets can also actively migrate across the inflamed vascular wall in response to a variety of microenvironmental and tumor factors.
- Additional critical receptors that can impact interactions between platelets and tumor include those influencing immunomodulation.
- Platelets serve as "First Responders" in the complete cancer continuum.

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Platelets and Inflammatory Disorders of Connective Tissue

Jerry Ware and Steven R. Post

Abstract

The circulating blood platelet as a participant in inflammatory pathways has been the focus of intense investigation and the topic of several reviews and other chapters in this book (Franco et al. Blood. 126:582–588, 2015). Inflammation underlies the pathophysiologic response in many diseases, and therefore defining the role of the platelet in inflammatory responses is paramount to understanding the molecular basis of the disease process (Leslie. Science 328(5978):562–564, 2010). In this chapter, we focus on the historical background describing the role of platelets in connective tissue inflammation, with emphasis on studies linking platelets and rheumatoid arthritis. A link between platelets and the inflammatory response starts with an appreciation for platelets being a specific cellular entity restricted to higher mammals. Lower vertebrates, such as fish and birds, do not have platelets, but instead have a dual-functioning cell, the thrombocyte, that has both immune and hemostatic properties (see Momi and Wiwanitkit 2017). Thus, the divergence of thrombocyte function to multiple cell types, such as neutrophils, monocytes, and platelets, might be expected to result in functional overlap with some similar properties retained by each individual cell type in higher species. The participation of platelets in inflammation needs careful consideration given the widespread use of antithrombotic agents for prevention of cardiovascular events. Understanding the molecular basis of the platelet/inflammatory axis will also ensure the best practice decisions will be made for the treatment of cardiovascular disease and additional inflammatory conditions.

Platelets and Synovial Fluid

Two studies in 1978 surprisingly identified platelet antigen in the synovial fluid of patients with rheumatoid arthritis (Ginsberg et al. 1978; Yaron and Djaldetti 1978). At this time, the biochemical analyses of platelet function were primarily restricted to understanding the role of platelets in hemostasis and thrombosis. With regard to platelet antigen and synovial fluid, it was unclear whether the platelet contributed to the worsening of the disease or had been hijacked for its wound healing properties within a joint undergoing pathologic destruction. As an active participant in vessel healing, the platelet is well known to contain storage granules containing a plethora of growth factors that can stimulate tissue remodeling. So, while platelets were identified in the synovial fluid, whether they were the "yin" or "yang" of the inflammatory process was unknown (Mueller 2013).

A subsequent study in 1981 again reported platelet antigen in the synovial fluid of all 13 patients examined with adult rheumatoid arthritis (Endresen 1981). In contrast, this study reported a near absence of platelets in the synovial fluid from patients with osteoarthritis. Importantly, a positive correlation between the number of platelet aggregates

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and the number of lymphocytes in synovial fluid was also observed (Endresen 1981). Thus, a link between platelets and an autoimmune response began to emerge, but it was not clear whether platelets were impacting rheumatoid arthritis in a positive or negative manner. A larger study followed in 1984 and again confirmed the presence of platelet antigen in the synovial fluid of 110 patients with various forms of arthritis (Farr et al. 1984). Of note, 50/50 patients with rheumatoid arthritis had platelet antigen in the synovial fluid (Farr et al. 1984) In addition, these investigators noted a direct link between circulating platelet count and higher level of platelet antigen in synovial fluid (Farr et al. 1984). Subsequent studies tended to focus on the release of growth factors from the platelet with little consideration as to whether the platelet's major effect in rheumatoid arthritis was "yin," "yang," or both (Endresen and Forre 1992).

Platelet Microparticles in the Synovial Space

Studies in the 1970s and 1980s established the presence of platelet antigen in synovial fluid, but it was not until much later that platelet microparticles were identified as the major contributor to the presence of platelet antigen in synovial fluid (Boilard et al. 2010a).

This observation was based on a careful examination of the platelet antigen and its fluorescence associated with structures smaller than expected for platelets and appearing to be in close proximity, or attached, to neutrophils. Flow cytometry studies confirmed a high level of CD41+ microparticles in the synovial fluid of patients with rheumatoid arthritis, which was not the case in an overwhelming majority of patients with a diagnosis of osteoarthritis (Fig. 1). The microparticles derived from platelets were the most abundant microparticle in synovial fluid supporting a connection between platelets and the pathology associated with rheumatoid arthritis.

The platelet microparticle is a membrane-derived particle released following some level of platelet activation (see Cointe et al. 2017) (Ardoin et al. 2007; Italiano et al. 2010; Pisetsky et al. 2012). It was the laboratory of David Lee (working at Brigham and Women's Hospital in Boston) who made the observation that platelet-specific antigen present in the synovial fluid of rheumatoid arthritis patients was largely restricted to fluorescent microparticles (Fig. 1). Whether the microparticles are generated by intact platelets within the synovial space or generated within the bloodstream and then migrate to the synovial space via interaction with leukocytes is still unknown.

Several mouse models of rheumatoid arthritis exist (Asquith et al. 2009). One of the more widely used models, the K/BxN serum transfer model, mimics many of the salient features of human autoimmune rheumatoid arthritis (Kouskoff et al. 1996). Generating severely thrombocytopenic mice in the K/BxN model established a direct link between joint inflammation and the presence of circulating platelets (Fig. 2) (Boilard et al. 2010a). By examining mice that were deficient in the expression of different platelet proteins, these investigators identified platelet surface antigens that could influence microparticle release. Genetic deficiency of platelet glycoprotein (GP) Ib-IX, the β3 integrin, thromboxane synthase, and COX-1 showed no noticeable difference in the development of arthritis using the K/BxN serum transfer model. In contrast, the absence of the murine platelet GPVI receptor reduced the arthritic phenotype, an effect that was correlated with the reduced ability of exogenous agonists, such as collagen, to stimulate platelet microparticle release (Boilard et al. 2010a). Although the mice still developed an inflammatory joint, the severity of

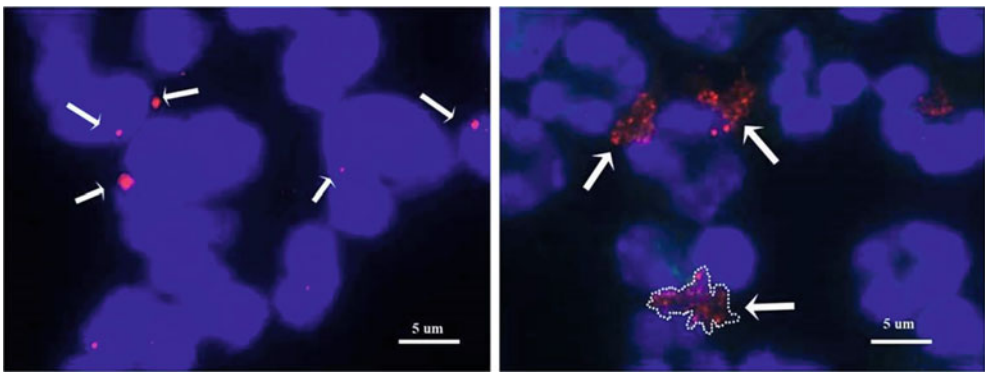

Fig. 1 Cytospin preparations of rheumatoid arthritis leukocytes either alone (*left*) or spiked with intact platelets from peripheral blood (*right*) were labeled with nuclear dye. Draq5 (*blue*) and stained with a platelet marker anti-CD41 (*red*). *Arrows* on the left identify punctuate platelet-specific microparticles of <1 μm diameter found associated with leukocytes, whereas *arrows* on the right identify intact platelets (2–4 μm diameter) spiked into the cell population, one of which is outlined with a *dotted line* [*from Boilard et al. (2010a, b). Reprinted with permission from AAAS*]

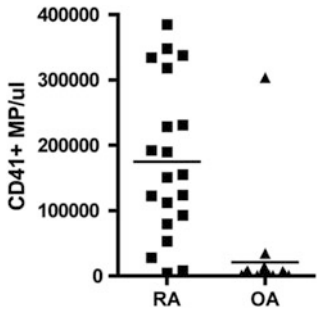

Fig. 2 Flow cytometric quantification of platelet microparticles (<1 mm as determined by size calibration beads) in rheumatoid arthritis (RA) and osteoarthritis (OA) synovial fluid (SF) after removal of leukocytes by centrifugation ($n = 20$ donors per group) [*from Boilard et al*. (2010a, b). *Reprinted with permission from AAAS*]

inflammation was significantly reduced supporting the conclusion that platelet GPVI and the GPVI-dependent release of platelet microparticles contribute to the inflammatory response of rheumatoid arthritis (Zimmerman and Weyrich 2010).

The extent to which these observations made in murine models of rheumatoid arthritis translate to human rheumatoid arthritis has still not been determined. Moreover, characterization of the human GPVI gene has revealed genetic haplotypes corresponding to altered in vitro platelet responses (Trifiro et al. 2009). A growing body of evidence has linked these haplotypes to pathologic responses in acute clinical settings including myocardial infarction and stroke (Bigalke et al. 2010a, b; Snoep et al. 2010). The haplotypes, referred to as *GP6a* and *GP6b*, differ in five codons resulting in three polymorphic amino acids in the extracellular portion of the GPVI (S219P, K237E, and T249A) and two polymorphic amino acids in the cytoplasmic tail (Q317L and H322N) (Trifiro et al. 2009). Relevant to platelet function, the polymorphisms associated with *GP6b* were shown to reduce the cytoplasmic interaction between the GPVI and Fyn/Lyn and attenuate the rate and extent of Syk phosphorylation (Trifiro et al. 2009). Whether these polymorphisms in GPVI affect the development of autoimmune disease, specifically rheumatoid arthritis, remains to be studied.

Platelet-Rich Plasma and Treatment of Osteoarthritis

Several studies demonstrate significant improvement using platelet-rich plasma in the treatment of osteoarthritis (see Alsousou and Harrison 2017). In defining the "yin-yang" relevance of platelets in the inflammatory response, the distinction between rheumatoid arthritis and osteoarthritis becomes important. Osteoarthritis is the more common of the two diseases and is typically the result of mechanical wear and tear on joints. In contrast, rheumatoid arthritis is the outcome of an autoimmune disease in which joints are being damaged by activation of an immune response. Thus, capitalizing on the platelet's wound healing properties seems logical for treating osteoarthritis, and the use of an autologous cocktail of growth factors represents a simple and straightforward nonsurgical alternative for repairing cartilage injuries (Xie et al. 2014). Based solely on studies of patients with rheumatoid arthritis, it would seem that platelet-rich plasma might be harmful when administered to the diseased joint, but this has yet to be examined. This does, however, highlight the very distinct pathophysiologic pathways contributing to rheumatoid arthritis and osteoarthritis.

The pro-inflammatory role of the platelet in rheumatoid arthritis and the repairing function of the platelet in osteoarthritis magnify the importance of making a definitive diagnosis in joint disease. Presumably, the pro-inflammatory properties of the platelet and/or platelet microparticles that worsen rheumatoid arthritis are also present in osteoarthritis. The storage granule cargo of the platelet is loaded with both pro-inflammatory and anti-inflammatory mediators (see chapter "Platelets and the Immune System"). In the case of administering platelet-rich plasma to a diseased joint, the key is likely the relative concentrations of the various pro- versus anti-inflammatory mediators. Studies have shown that some anti-inflammatory proteins are present at higher concentrations within the platelet than their corresponding pro-inflammatory counterparts (Woodell-May et al. 2011). For example, the concentration of the IL-1 receptor antagonist (IL-1Ra) is 23,000 times higher than that of IL-1α and 8,000 times higher than IL-1β (Woodell-May et al. 2011). This likely explains the pro-repair properties of platelet-rich plasma when used in the treatment of patients with osteoarthritis. The relative balance of pro- versus anti-inflammatory proteins in microparticles is not clear. Thus it might be speculated that concentrations of IL-1, which is considered to be a pro-inflammatory mediator in models of rheumatoid arthritis, are higher in platelet microparticles and may therefore contribute to the pathophysiology of autoimmune joint disease. This could be analogous to the proposed selective release of alpha granule components during platelet activation (Italiano et al. 2008), but clearly, more definitive studies investigating the mechanisms involved are warranted.

Platelet-Leukocyte Axis

Specific interactions between platelets and leukocytes (e.g., monocytes and neutrophils) represent a key axis in understanding the major contribution that platelets play in arthritic diseases. On a larger scale, this axis may represent one of the least understood but critically important

interactions impacting a wide range of inflammatory disease processes (Franco et al. 2015). As discussed in other chapters (see Evangelista et al. 2017), there is a growing interest in identifying the key receptors and ligands involved in platelet-leukocyte interactions and the consequences of altering this axis on the initiation and progression of disease. From this standpoint, the work that has been done, to date, describing platelet-dependent contributions to rheumatoid arthritis is an excellent starting point to better understand the role platelets play in the pathophysiology of rheumatoid arthritis and other chronic inflammatory disorders.

Antithrombotic Agents and Joint Inflammation

Given the role platelets play in a number of inflammatory diseases, it is important to fully understand the impact of the widely used platelet-specific antithrombotic drugs in patients with joint inflammation. This is particularly so given that the administration of clopidogrel in mouse models of rheumatoid arthritis worsens the disease (Boilard et al. 2010b). Agrawal et al. also observed a link between clopidogrel and arthritis in a rat model of peptidoglycan polysaccharide-induced arthritis (Garcia et al. 2011). Determining if these rodent observations translate to the human disease is important as even following its patent expiration, clopidogrel continues to be one of the most widely prescribed and best selling drugs in the world (Topol and Schork 2011). Importantly, several human case studies have suggested that clopidogrel administration worsened acute arthritis (Agrawal et al. 2013; Garg et al. 2000). Some have argued that implicating the use of clopidogrel to worsening of the arthritis could cost lives if patients are reluctant to take their antithrombotic medication for the treatment of concomitant cardiovascular disease (Green et al. 2002). Interestingly, some studies report that prasugrel, which like clopidogrel is a prodrug whose active metabolite blocks the $P2Y_{12}$ receptor, does not have a pro-arthritic effect (Agrawal et al. 2013). Clearly, more work is needed at the mechanistic level to understand the relationship between these widely prescribed drugs and the potential pro-inflammatory outcomes. Recently, it has been reported in allergic inflammatory models that there is a dichotomy of platelet activation via purinergic receptors with the P2Y1, a receptor being most important rather than $P2Y_{12}$ for pro-inflammatory actions of platelets (Amison et al. 2015).

Future Directions

The platelet's ability to modulate an inflammatory response places an abundance of pathophysiologic processes at the forefront of new and innovative investigations. The mechanistic basis of platelet microparticle formation and the influence on the diseased joint are likely to add paradigms easily applied to other immune-based diseases. Are the microparticles formed within the synovial space or do they migrate from the vasculature? It is easy to suggest collagen via activation of the GPVI receptor could drive microparticle formation, yet studies have also shown that laminin is also a GPVI ligand (Inoue et al. 2006). Platelets and neutrophils must presumably pass through a basement membrane region that separates the bloodstream from the synovial space. Other questions include does laminin, as a major protein of basement membranes, stimulate microparticle release?

From the clinical perspective, the large dataset information now being mined from patients who have undergone long-term treatment with antiplatelet drugs, such as aspirin and clopidogrel, is likely to be highly informative. Already, data has emerged linking aspirin with anticancer benefits (Rothwell et al. 2012a, 2012b). Perhaps some of this benefit is linked to the cancer/inflammation axis that is being recognized as a critical component of the tumor/stromal interaction. Likely, some of these interactions are playing a similar role in autoimmune diseases. Also relevant will be to examine whether the new class of direct $P2Y_{12}$ inhibitors are linked to worsening arthritic diseases, as has been described for clopidogrel. This information will be important both for the basic scientist unraveling the cellular and molecular mechanisms at play and also for the practicing clinician trying to provide guidance in the treatment of management of disease. Antiplatelet therapies for cardiovascular disease will continue to be important and lifesaving therapies, but how they affect other ongoing disease processes will need to be considered. So while the platelet's role in hemostasis and thrombosis could be considered a mature field and understood with great molecular detail, the future needs to take these same paradigms and apply them to the vast array of pathologies where the platelet may not be causing the disease but does have a significant impact on severity and how the disease is managed (Leslie, 2010).

Take Home Messages

- Platelets participate in the "yin and yang" of connective tissue inflammation.
- Platelet antigen is found in the synovial fluid of patients with rheumatoid arthritis.
- Release and sequestration of platelet microparticles to the synovial space worsens rheumatoid arthritis.
- Platelet-rich plasma can be therapeutic wound healer in osteoarthritis.
- The platelet/leukocyte axis may impact a wide range of inflammatory disease processes.
- Antithrombotic therapies can impact inflammatory diseases.

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Platelets and Skin Disorders

Risa Tamagawa-Mineoka

Abstract

Recent accumulating evidence indicates that platelets act as effector cells in initiating and modulating inflammatory and immune responses in various organs including the skin. On activation in response to various factors such as chemokines and microbial toxins, platelets express adhesive and immune receptors such as P-selectin and Toll-like receptors on their surface and release soluble mediators such as chemokines, cytokines, and antimicrobial peptides. In patients with inflammatory skin disorders such as atopic dermatitis and psoriasis, platelets circulate in an activated state, and the degree of platelet activation correlates with disease severity. Platelets are deeply involved in the pathogenesis of several inflammatory skin disorders such as atopic dermatitis, contact dermatitis, psoriasis, urticaria, and infectious diseases via formation of platelet-leukocyte complexes, releasing chemotactic factors, inhibiting monocytic apoptosis, inducing neutrophil phagocytosis, promoting allergic sensitization, and releasing antimicrobial peptides.

Introduction

The skin is the largest organ of the human body that is in direct contact with the outside environment. The skin plays a key role in prevention of permeation or loss of water and other molecules, temperature regulation, and sensation. In addition, the skin functions as an important tissue in immune reactions by protecting the body from microbes and harmful external influences. Recent accumulating evidence indicates that platelets are involved in the development of inflammatory and immune processes in several organs including the skin, in addition to their well-established role in hemostasis and coagulation. The aim of this chapter is to review the involvement of platelets in the mechanisms of inflammatory and immune responses in the skin.

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The Role of Platelets in the Pathogenesis of Atopic Dermatitis

Atopic dermatitis is a chronic, relapsing inflammatory skin disease characterized by epidermal barrier dysfunction, pruritus eczematous lesions, increased levels of T helper type 2 (Th2) cytokines such as interleukin (IL)-4 and IL-13, high serum IgE levels, positive immediate hypersensitivity to food or environmental allergens, and peripheral blood eosinophilia (Leung 2013). Interestingly, plasma levels of platelet-derived chemokines such as platelet factor-4 (PF-4, CXCL4) and β-thromboglobulin (β-TG, CXCL7), which are known as platelet activation markers, are greatly increased in patients with atopic dermatitis compared with those in healthy individuals (Tamagawa-Mineoka et al. 2008, 2009a, b; Nastalek et al. 2011). In addition, the levels of these chemokines correlate with disease severity (Tamagawa-Mineoka et al. 2008). Moreover, plasma levels of other platelet activation markers including platelet-derived microparticles, vascular endothelial growth factor, and platelet-derived growth factor are also elevated in atopic dermatitis patients compared to healthy controls

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(Tamagawa-Mineoka et al. 2009a, b; Nastalek et al. 2011). These findings suggest that platelets circulate in an activated state in patients with atopic dermatitis and are involved in the pathogenesis of this disease. On the other hand, platelet aggregation induced by thrombin, adenosine diphosphate (ADP), and collagen is not increased in patients with atopic dermatitis compared with those in healthy controls (Rogala et al. 1999). Therefore, in atopic dermatitis, although the hemostatic function of platelets, such as the ability to aggregate, is not impaired, various inflammatory stimuli may activate platelets in circulating blood. It has been demonstrated that the incidence of ischemic stroke is increased in patients with atopic dermatitis (Su et al. 2014), implying that platelet activation may be possibly related to the pathogenesis of atopic dermatitis.

Platelets usually circulate in a resting state in blood and can be activated by a range of immunological and non-immunological stimuli including thrombin, ADP, collagen, chemokines, platelet-activating factor (PAF), thromboxane A2, lipopolysaccharide, other microbial toxins, and pathogens (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). Platelets express numerous kinds of chemokine receptor, CCR (CCR1, CCR2, CCR3, CCR4, CCR5, CCD6, CCR7, CCR8, CCR9, and CCR10) and CXCR (CXCR1, CXCR2, CXCR3, CXCR4, and CXCR5) on their surface (Gear and Camerini 2003; von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). Several chemokines such as stromal cell-derived factor-1 (SDF-1, CXCL12) and thymus and activation-regulated chemokine (TARC, CCL17) induce platelet aggregation with ADP or thrombin, and platelet chemokines themselves can directly activate platelet function. Platelets also express both high-affinity IgE (FcεRI) (Hasegawa et al. 1999) and low-affinity IgE (FcεRII) receptors (Joseph et al. 1986). Engagement of FcεRI on platelets induces the release of serotonin (5-hydroxytryptamine, 5-HT) and regulated on activation, normal T cell expressed and secreted (RANTES, CCL5) (Hasegawa et al. 1999). These findings suggest that platelets may be activated via allergen or various soluble mediators such as chemokines. Moreover, patients with atopic dermatitis often scratch their skin due to severe itching. Therefore, platelet activation may occur by coagulation of blood on the scratched skin.

In a murine model of chronic allergic dermatitis induced by repeated allergen application that resembles some of the features of atopic dermatitis such as increased levels of Th2 cytokines in inflamed skin and elevated levels of serum IgE (Kitagaki et al. 1995, 1997), depletion of platelets circulating in blood greatly reduces the degree of skin inflammation as determined by skin thickness and leukocyte infiltration in inflamed skin (Tamagawa-Mineoka et al. 2007). Furthermore, the elevation of serum IgE levels and gene expression of Th2 in inflamed skin is decreased in platelet-depleted mice. On the other hand, the immediate-type hypersensitivity reaction is an important component of the pathogenesis of atopic dermatitis (Leung 2013). The mice that are sensitized and then challenged with the major egg white protein, ovalbumin, develop on immediate-type hypersensitivity reaction characterized by biphasic responses: early-phase and late-phase reactions. Although the early-phase reaction is not affected by platelet depletion, the late-phase reaction is reduced in thrombocytopenic conditions (Tamagawa-Mineoka et al. 2009a, b). In both murine models of chronic allergic inflammation and the immediate-type hypersensitivity reaction, cutaneous inflammation is increased by platelet infusion in platelet-depleted mice (Tamagawa-Mineoka et al. 2007, 2009a, b). These findings indicate that platelets are important for the development of cutaneous allergic inflammation.

Platelets contain a wide variety of mediators contributing to inflammation in three major types of storage granules: α-granules, dense granules, and lysosomes (Table 1) (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). Platelet

Table 1 Granules contents

α-granules
Chemokines
See Table 2
Growth factors
Platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, insulin-like growth factor
Cytokines
IL-1β, IL-33
Lipid mediators
PAF, prostaglandin E2, microparticles
Antibacterial proteins
Thrombocidins 1 and 2
Adhesion molecules
P-selectin, CD40 ligand, integrins (α2β1, α5β1, α6β1, αLβ2, $\alpha_{IIb}\beta_3$, αvβ3), thrombospondin, fibrinogen, fibronectin
Others
Albumin, IgG, IgM, IgA
Dense granule
ADP, adenosine triphosphate, Ca^{2+}, Mg^{2+}, polyphosphate, glutamate, serotonin, histamine
Lysosomal granule
β-Glucuronidase, β-N-acetylglucosaminidase, cathepsin D and E, lysosomal membrane proteins (LAMP-1, LAMP-2, and CD63)

The table is adapted and expanded from the references Tamagawa-Mineoka (2015) and Tamagawa-Mineoka and Katoh (2016), with permission

Table 2 Platelet-derived chemokines

Chemokines	Cellular targets
CXC chemokine	
CXCL1 (growth-related oncogene-α)	Neutrophil
CXCL4 (PF-4)	Monocyte, neutrophil, T cell
CXCL5 (epithelial neutrophil-activating peptide-78)	Neutrophil
CXCL7 (β-TG, neutrophil-activating peptide-2)	Neutrophil, macrophage
CXCL8 (IL-8)	Neutrophil
CXCL12 (SDF-1)	T cell, monocyte
CC chemokine	
CCL1 (I-309)	T cell
CCL2 (macrophage chemotactic protein-1)	Monocyte
CCL3 (macrophage inflammatory protein-1α)	Monocyte, neutrophil, eosinophil
CCL5 (RANTES)	Monocyte, T cell, eosinophil
CCL7 (macrophage chemotactic protein-3)	Monocyte, T cell, eosinophil
CCL17 (TARC)	T cell

The table is adapted from the reference Tamagawa-Mineoka (2015), with permission

α-granules contain numerous chemokines, which are a superfamily of potent leukocyte chemotactic cytokines, and rapidly secrete mediators upon activation (Table 2) (Gear and Camerini 2003; von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). It has been demonstrated that the gene expression of PF-4 is elevated in a mouse model of atopic dermatitis (Watanabe et al. 1999). PF-4 induces adhesion and chemotaxis of eosinophils (Bruijnzeel et al. 1993; Hayashi et al. 1994), which are often increased in circulating blood and in inflamed skin of atopic dermatitis. In addition, on activation, platelets from atopic dermatitis patients release a great amount of TARC (Fujisawa et al. 2002) which is deeply involved in the pathogenesis of atopic dermatitis (Leung 2013). In a hapten-induced atopic dermatitis murine model, injection of activated platelet supernatant into inflamed skin results in increased leukocyte infiltration, which is not blocked by pretreating platelets with neutralizing macrophage inflammatory protein-1α (MIP-1α, CCL3), RANTES, and TARC in the platelet supernatant (Tamagawa-Mineoka et al. 2007). These findings indicate that platelet-derived chemokines can induce leukocyte recruitment into inflamed skin lesions of atopic dermatitis.

In the chronic lesions of atopic dermatitis, there is dense infiltration of monocytes/macrophages. While unstimulated monocytes undergo apoptosis, apoptosis of monocytes is inhibited via stimulation by several inflammatory mediators (Katoh et al. 2000; Soga et al. 2007a). In addition, monocytes from patients with atopic dermatitis show a lower rate of apoptosis in vitro than those from healthy controls (Soga et al. 2007a). These findings suggest that monocytes/macrophages stimulated in the process of inflammation can prolong the survival and be related to the development of chronic inflammation. 5-HT, a monoamine neurotransmitter, is a major component of platelet dense granules as well as mast cell granules (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). 5-HT prevents apoptosis of monocytes (Soga et al. 2007a) and promotes monocyte differentiation into dendritic cells (Katoh et al. 2006). In addition, PF-4 promotes monocyte survival via suppression of apoptosis and induces monocyte differentiation into macrophages (Scheuerer et al. 2000). Moreover, apoptosis of monocytes can be suppressed by phagocytosis of platelets (Lang et al. 2002). These findings suggest that platelets may contribute to the establishment of chronic dermatitis by suppression of monocyte apoptosis.

Platelets also release important cytokines including IL-33 (Unno et al. 2014, 2015), which can activate Th2 cells, mast cells, and eosinophils and promote Th2-type immune responses (Moritz et al. 1998; Xu et al. 1998; O' Neill LA, Dinarello CA 2000; Schmitz et al. 2005). In patients with atopic dermatitis, serum IL-33 levels are greatly increased compared with those in healthy individuals (Tamagawa-Mineoka et al. 2014). Although it has been reported that the expression of IL-33 is increased in keratinocytes, endothelial cells, and fibroblasts of skin lesions in patients with atopic dermatitis (Pushparaj et al. 2009; Savinko et al. 2012), platelet-derived IL-33 may be also involved in the development of Th2 inflammation in atopic dermatitis. In addition, arachidonic acid metabolites are also important mediators in allergic skin inflammation. It has been demonstrated that platelets from patients with atopic dermatitis release a large amount of 12-hydroxyeicosatetraenoic acids (12-HETE) (Hilger et al. 1991). Stimulation of platelets with *Staphylococcus aureus*, which often colonizes the skin of atopic dermatitis patients, promotes the release of 12-HETE from platelets (Neuber et al. 1992). Therefore, platelet-derived arachidonic acid metabolites may be also related to the development of cutaneous inflammation.

Platelets express various kinds of functional surface receptors (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). Activated platelets are capable of increasing leukocyte rolling in cutaneous postcapillary venules, via the aggregate formation with leukocytes (Fig. 1) through a distinct set of adhesion molecules (Fig. 2) (Ludwig et al. 2004;

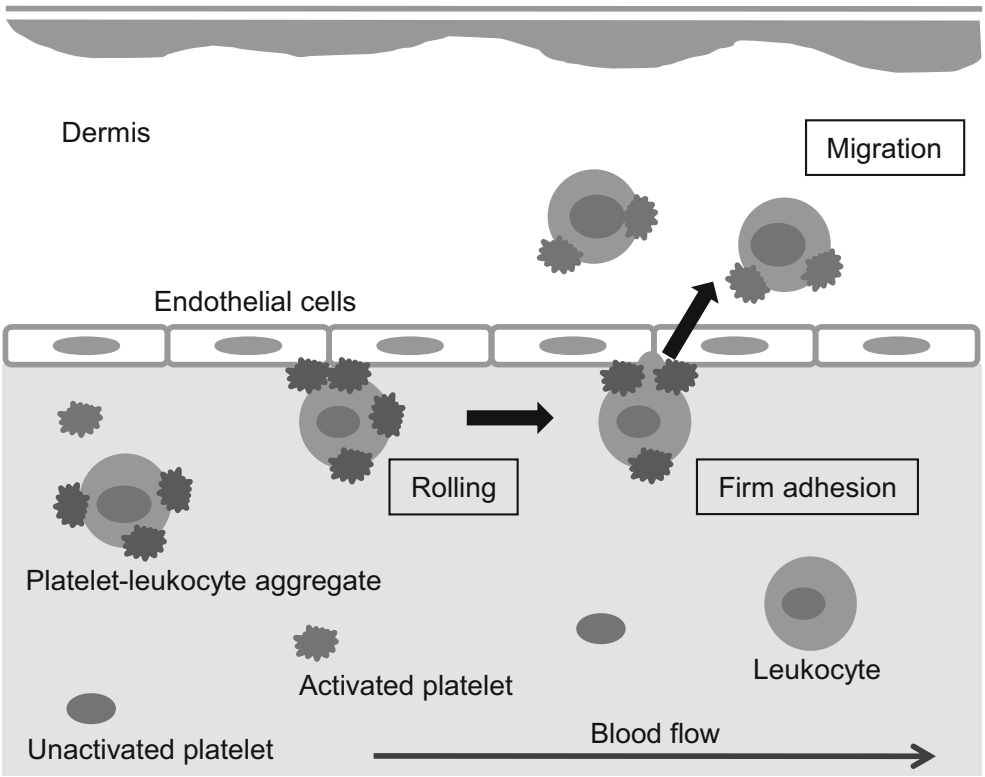

Fig. 1 Role of platelets in leukocyte recruitment from blood to the skin tissue. On activation, platelets bind leukocytes via several adhesion molecules in circulating blood. The complexes of platelets and leukocytes roll along the endothelium and transmigrate into subendothelial tissue. The figure is adapted from references Tamagawa-Mineoka (2015) and Tamagawa-Mineoka and Katoh (2016), with permission

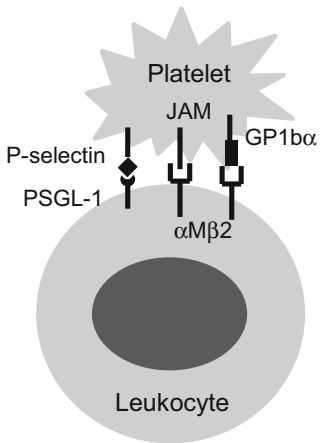

Fig. 2 Interaction of platelets with leukocytes via adhesion molecules. The interaction between platelet P-selectin and leukocyte PSGL-1 is the first step of complex formation, leading to the activation of integrins on the leukocyte surface and the firm adhesion of leukocytes to activated platelets. The figure is adapted from references Tamagawa-Mineoka (2015) and Tamagawa-Mineoka and Katoh (2016), with permission. *JAM* junctional adhesion molecule

von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). P-selectin (CD62P) is one of the receptors that are important for formation of platelet-leukocyte complexes (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). On activation, platelets express P-selectin on their membranes or secrete this into plasma. By binding its major ligand, P-selectin glycoprotein-1 (PSGL-1), which is expressed on almost all kinds of leukocyte, platelet P-selectin supports the adhesion of polymorphonuclear cells, monocytes/macrophages, and lymphocytes (von Hundelshausen and Weber 2007; Kasperska-Zajac et al. 2008; Katoh 2009; Semple et al. 2011; Tamagawa-Mineoka 2015; Tamagawa-Mineoka and Katoh 2016). In murine models of chronic allergic inflammation and the immediate-type hypersensitivity reaction, P-selectin expression on platelets and an increased number of platelet-leukocyte complexes are increased in circulating blood. Interestingly, leukocyte infiltration into inflamed skin is increased by platelet infusion in thrombocytopenic conditions of these allergic dermatitis models, but is not restored by infusion of P-selectin-deficient platelets (Tamagawa-Mineoka et al. 2007, 2009a, b). These finding suggest that platelet P-selectin is important in formation of platelet-leukocyte aggregates and transmigration of them into inflamed skin tissue in atopic dermatitis.

The Role of Platelets in the Pathogenesis of Allergic and Irritant Contact Dermatitis

Allergic contact dermatitis is a common allergic inflammatory skin disorder characterized by eczematous lesions and depends on the delay-type hypersensitivity reaction. The contact hypersensitivity response consists of two phases:

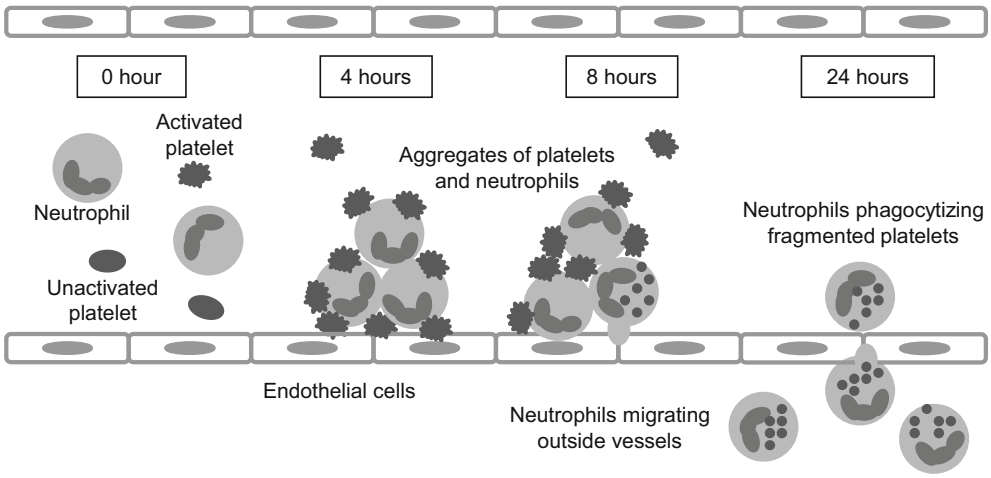

Fig. 3 Platelet interactions with neutrophils and the endothelium in the contact hypersensitivity response. Aggregates of platelets and neutrophils adhere to endothelial cells within the vessels at 4 h after allergen challenge. At 8 h, neutrophils ingesting fragmented platelets are found inside the vessels. At 24 h, the number of neutrophils ingesting fragmented platelets outside the vessels increases. The figure is adapted from references Daito et al. (2014) and Tamagawa-Mineoka (2015)

sensitization and elicitation. Deficiency of platelet P-selectin impairs the sensitization process in the contact hypersensitivity responses (Ludwig et al. 2010). On the other hand, the contact hypersensitivity responses are decreased after depletion of blood platelets in sensitized mice (Tamagawa-Mineoka et al. 2007), suggesting that platelets are also involved in the elicitation process. Furthermore, it has been demonstrated that platelets are activated via FcεRI and initiate the contact hypersensitivity responses by releasing 5-HT (Matsuda et al. 1997). These findings suggest that platelets are involved in both the sensitization and elicitation phases of the contact hypersensitivity responses by expressing immune-related receptors and releasing inflammatory mediators.

Neutrophils are the most abundant leukocytes in circulating blood. They migrate not only to sites of infection but also to the skin lesions in a mouse model of allergic contact dermatitis. It has been demonstrated that platelets bind to leukocytes, and the complexes of platelets and neutrophils adhere to endothelial cells within blood vessels at 4 h after allergen challenge in the contact hypersensitivity responses (Fig. 3) (Daito et al. 2014). At 4 to 8 h after challenge, neutrophils begin to phagocytose platelets, and neutrophils including fragmented platelets are found inside the vessels. At 24 h after elicitation, when the contact hypersensitivity responses peak, the number of neutrophils including fragmented platelets outside the vessels increases. These findings suggest that the process of phagocytization of platelets by neutrophils is related to leukocyte transmigration from blood to subendothelial tissue in allergic contact dermatitis.

In contrast to allergic contact dermatitis, irritant contact dermatitis occurs as a consequence of the direct action of chemical agents and shows non-antigen-specific cutaneous responses (Corsini and Galli 1988). The irritant reaction is characterized by edema and leukocyte infiltration in the skin. Depletion of platelets by an antiplatelet antibody decreases dermal edema in a murine model of irritant contact dermatitis, although leukocyte infiltration is not affected (Senaldi and Piguet 1997). Therefore, platelets also contribute to the pathogenesis of irritant contact dermatitis and are important for the development of edema, but not for leukocyte recruitment into inflamed sites.

Platelets are closely associated with other types of cutaneous immune reactions in addition to the immediate and delayed-type hypersensitivity reactions. Platelets induce recruitment of leukocytes into inflamed sites via the interaction of P-selectin on platelets interacting with PSGL-1 on leukocytes in a murine model of the cutaneous Arthus reaction (Hara et al. 2010). Moreover, platelets contribute to skin allograft rejection through the recruitment of T cells and increased inflammatory mediators (Xu et al. 2006; Swaim et al. 2010). These findings suggest that platelets initiate and sustain several types of immune reactions in the skin.

The Role of Platelets in the Pathogenesis of Psoriasis

Psoriasis is a chronic inflammatory immune-mediated disease characterized by scaly, red cutaneous plaques. Several epidemiological studies have demonstrated that psoriasis patients have an increased risk for developing thrombotic vascular diseases, including myocardial infarction, stroke (Kasperska-Zajac et al. 2005; Gelfand et al. 2006, 2009; Mehta et al. 2010; Boehncke et al. 2011), and thromboembolic events (Ahlehoff

et al. 2011). It has been demonstrated that chronic skin-specific inflammation promotes aortic inflammation and thrombosis in a murine model of psoriasiform skin disease (Wang et al. 2012). Recent studies have suggested that the persistent skin inflammation in psoriasis can develop into systemic inflammation which leads to dyslipidemia, insulin resistance, and endothelial dysfunction, resulting in thrombotic vascular diseases (Boehncke et al. 2011).

It has been also reported that psoriasis patients have platelet hyperaggregability. Platelet aggregation in vitro induced by thrombin or ADP is markedly increased in psoriasis patients compared with that in healthy individuals, and this elevated platelet aggregation is greatly reduced following improvement of psoriatic skin lesions (Hayashi et al. 1985; Chandrashekar et al. 2015). In addition, platelet activation markers such as platelet-derived chemokines, soluble P-selectin, and platelet-derived microparticles are also significantly elevated in blood of patients with psoriasis (Tamagawa-Mineoka et al. 2009a, b, 2010; Chandrashekar et al. 2015). These findings suggest that blood platelets are activated in patients with psoriasis. In addition to platelet-derived soluble mediators, mean platelet volume (MPV) and platelet distribution width (PDW) are regarded as indicators of platelet activation. Patients with psoriasis and psoriatic arthritis have higher levels of MPV and PDW in comparison with controls (Canpolat et al. 2010; Chandrashekar et al. 2015). Platelet volume is closely related to platelet function with large platelets producing a greater amount of soluble mediators such as thromboxane B2 or serotonin, and they express numerous adhesion molecules (Thompson et al. 1982; Martin et al. 1983). Furthermore, it has been reported that the expression of P-selectin on blood platelets is increased in patients with psoriasis in parallel with disease severity (Ludwig et al. 2004). Therefore, activated large platelets play important roles in the pathogenesis of psoriasis by releasing great quantities of mediators and adhesion molecules that may contribute to the development of thrombotic vascular diseases.

The Role of Platelets in the Pathogenesis of Urticaria

Urticaria is a common skin disease characterized by wheals, in which mast cell activation leads to acute inflammatory responses and with vasodilatation and increased permeability of small blood vessels. Several studies have been performed to assess platelet function in patients with urticaria. It has been reported that plasma levels of platelet activation markers including PF-4, β-TG, and soluble P-selectin are not different between patients with chronic idiopathic urticaria and control subjects (Kasperska-Zajac et al. 2005; Tamagawa-Mineoka et al. 2010). On the other hand, it has been demonstrated that in chronic urticaria patients with positive autologous serum skin test, MPV is greatly increased compared with chronic urticaria patients with negative autologous serum skin test or control subjects (Magen et al. 2010). In addition, MPV in the patients with positive autologous serum skin test correlates with the disease severity score. In patients suffering from cold urticaria, plasma levels of PF-4 are enhanced following a cold challenge test (Wasserman and Ginsberg 1984). Furthermore, packed platelets are histologically seen within vessel lumen in cold urticaria (Lawlor et al. 1989). Some previous studies have also demonstrated that the coagulation cascade is activated in chronic urticaria, leading to thrombin production (Lundblad and White 2005; Asero et al. 2007). Therefore, platelet activation may occur via activation of the coagulation cascade and may contribute to the mechanisms in some types of urticarial reactions.

The Role of Platelets in the Pathogenesis of Infectious Diseases

The skin has direct contact with the external environment, and there are, therefore, numerous opportunities to interact with various pathogens such as bacteria and viruses. Platelets are involved in innate immunity via interactions with pathogens (Semple et al. 2011). They express many Toll-like receptor (TLR) family members including TLR1, TLR2, TLR4, TLR6, and TLR9 (Shiraki et al. 2004; Blair et al. 2009). Platelet-expressed TLR2 (Aslam et al. 2006; Beaulieu et al. 2011) and TLR4 (Aslam et al. 2006; Blair et al. 2009) have important roles in inflammatory responses such as bacterial infections. Stimulation of platelets with bacteria via TLR2 induces aggregation, expression of P-selectin, and formation of platelet-neutrophil complexes (Beaulieu et al. 2011). In addition, stimulation of megakaryocytes via TLR2 leads to the production of platelets with increased proinflammatory gene and protein expression (Aslam et al. 2006). On stimulation via TLR4, platelets induce neutrophil activation and formation of neutrophil extracellular traps that contain proteolytic activity, leading to killing the bacteria (Clark et al. 2007).

Furthermore, platelets contain antimicrobial peptides such as β-defensins, thrombocidins, PF-4, RANTES, connective tissue-activating peptide-3, platelet basic protein, thymosin β-4, fibrinopeptide B, and fibrinopeptide A (Krijgsveld et al. 2000; Tang et al. 2002; Kraemer et al. 2011). Platelets can directly kill bacteria by releasing such antimicrobicidal peptides or by phagocytozing bacteria (Rogala et al. 1999; Krijgsveld et al. 2000; Tang et al. 2002; Kraemer et al. 2011). For example, platelet-derived β-defensins impair the growth of bacteria and induce neutrophil extracellular trap formation (Kraemer et al. 2011).

Many platelet-derived antimicrobial peptides are associated with the CC and CXC chemokine family and can induce recruitment of leukocytes to sites of infection in addition to their antimicrobial functions. In addition to bacterial infections, platelets are associated with infections with human immunodeficiency virus, influenza, and malaria (Morrell et al. 2014). It has been demonstrated that TLRs are deeply involved not only in infectious disorders but also in noninfectious inflammatory diseases including contact hypersensitivity responses (Nakamura et al. 2015). Therefore, platelets expressing TLRs may be possibly related to the pathogenesis of noninfectious inflammatory skin disorders.

Take-Home Messages

- Platelets circulating in blood are activated in several inflammatory diseases such as atopic dermatitis, psoriasis, and urticaria.
- Activated platelets express adhesive and immune receptors such as P-selectin, CD40 ligand, and Toll-like receptors on their surface.
- Activated platelets release soluble mediators such as chemokines, cytokines, and antimicrobial peptides.
- Platelets are involved in the pathogenesis of cutaneous inflammatory diseases, by increasing leukocyte rolling on the endothelium and recruiting leukocytes into inflamed skin via release of inflammatory mediators.
- Phagocytization of platelets by neutrophils is related to leukocyte transmigration from blood to subendothelial tissue in allergic contact dermatitis.
- Platelets directly kill the bacteria by releasing such antimicrobicidal peptides or phagocytizing the bacteria.

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Platelets and Airway Diseases

Stefania Momi, Simon Pitchford, Paolo Gresele, and Clive P. Page

Abstract

The role of platelets as inflammatory cells is now well established. Given the peculiar characteristics of the lung circulation, with a broad capillary bed, platelets are especially involved with the physiology of the lungs and play a key role in a number of inflammatory lung disorders. The platelet precursors, megakaryocytes, are detected in the lung microcirculation; moreover platelets with their endothelium-protective and vascular reparative activities contribute to the lung capillary blood barrier integrity. Given the function of the lungs as first wall against pathogen invasion, platelets participate in immune defence of the normal lung. On the other hand, platelets may turn into effectors of the inflammatory reaction of the lungs to allergens, to infectious agents, to chemical agents and may contribute strongly to the perpetuation of chronic inflammatory reactions, largely by their ability to interact with other inflammatory cells and the endothelium. In this chapter we provide an overview of the role of platelets in several inflammatory lung disorders discussing the pathophysiologic bases of platelet involvement in these conditions and the experimental and clinical evidence for a role of platelets in lung diseases.

Platelets have a well-established role in haemostasis and thrombosis, but there is now considerable evidence that they also play an important role in a number of inflammatory diseases (see Ware and Post 2017), including respiratory diseases such as asthma (Pitchford and Page, Clin Exp Allergy 36: 399–401, 2006) and chronic obstructive pulmonary disease (COPD) (Ferroni et al. J Investig Med 48:21–27, 2000). There is a relevant amount of information on the involvement of platelets in allergic inflammatory diseases, and platelet abnormalities in patients with allergy have been reported for more than 40 years following the seminal observation by Benveniste et al. in 1972 that leukocyte-dependent histamine release from platelets involved IgE-mediated activation (Benveniste et al. J Exp Med. 136:1356–77, 1972). This work led to the discovery of the lipid mediator platelet-activating factor (PAF) capable of inducing eosinophilia (Arnoux et al. Am Rev Resp Dis 137: 855–860, 1988). It is becoming increasingly apparent that platelets are central to inflammatory diseases of the airways, being a source of inflammatory mediators (see Table 1) and spasmogens per se and being critical for the recruitment of leukocytes into tissues.

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Platelets in Asthma

Pathophysiologic Observations: Altered Platelet Functionality

- Platelets accumulate into the lungs upon allergen exposure.
- Platelet activation by allergen via an IgE-mediated process reveals an important causal involvement of platelets in the inflammatory response.
- Studies in animals reveal platelets are necessary for pulmonary leukocyte recruitment, chronic inflammatory events leading to airway remodelling, and the release of spasmogens to cause bronchoconstriction. Emerging reports suggest platelets are involved in initial allergen processing with antigen-presenting cells.
- Many of these processes can be attributable to a direct effect of platelets, in their capacity to migrate through lung tissue and be present in the localised environment.

In 1978, Gallagher and colleagues observed that platelets isolated from the peripheral blood of allergic patients during the allergy season often showed reduced secondary wave platelet aggregation, and it was suggested that they behaved like 'exhausted' platelets (Gallagher et al. 1978). Since this early observation, there have been many other reports of altered platelet behaviour in patients with allergic disease, ranging from altered arachidonic acid metabolism to greater turnover of intra-cellular signalling pathways (Audera et al. 1988; Block et al. 1990). Indeed, these platelet abnormalities may well account for the observation that patients with allergic asthma have a mild haemostatic defect and delayed thrombin generation detected using the template bleeding time (Szczeklik et al. 1986). This 'exhausted platelet' syndrome may be the consequence of continuous platelet activation in vivo and is compatible with the observation that allergic patients undergoing allergen provocation have a mild thrombocytopenia (Kowal et al. 2006; Sullivan et al. 2000; Maestrelli et al. 1990) with increased numbers of platelet–leukocyte aggregates in their peripheral blood. Such observations clearly support the conclusion that platelet activation accompanies allergic exposure in such patients (Gresele et al. 1982, 1993; Pitchford et al. 2003; Sladek et al. 1990). This may explain recent observations suggesting that patients with asthma often have co-morbidities, including cardiovascular disease (Cazzola et al. 2013) and an increased risk of pulmonary embolism (Majoor et al. 2013), particularly patients with more severe asthma possibly associated with use of glucocorticosteroids (Varas-Lorenzo et al. 2007). This hypothesis is further supported by a growing number of clinical observations showing increased markers of platelet activation in allergic asthmatics. These include RANTES (CCLS), platelet factor 4 (PF4), CXCL4 and β-thromboglobulin (β-TG, CXCL7) in the peripheral blood of patients with asthma (Maccia et al. 1977; Kowal et al. 2006; Yasuba et al. 1991; Knauer et al. 1981), as well the demonstration of increases in some of these platelet markers in bronchoalveolar lavage (BAL) fluid obtained from such patients (Jeffery et al. 1989; Metzger et al. 1987) (Table 1). Furthermore, there are other observations demonstrating extravascular platelets in the airways of both experimental animals (Pitchford et al. 2008) and in patients with asthma (Jeffery et al. 1989; Metzger et al. 1987) (see Petito et al. 2017).

In the context of respiratory diseases, it is of interest that platelets have long been recognised as a rich source of spasmogens (Arnoux et al. 1988; Yoshimi et al. 2001) that can lead to both contraction of airway smooth muscle in vitro (Inoue and Kannan 1989) and bronchoconstriction in vivo (Robertson and Page 1987; Yoshimi et al. 2001). Indeed, we have recently reported that a range of inflammatory mediators, exemplified by bradykinin, elicit airway obstruction in vivo that is platelet dependent, building on earlier observations of platelet involvement in airway obstruction (Keir et al. 2015; Coyle et al. 1993). However, perhaps of greater interest for respiratory diseases are the increasing numbers of observations demonstrating a crucial role for platelets in the recruitment of leukocytes into various tissues, including the lung (Pitchford et al. 2003, 2005; Kornerup et al. 2010). Platelet-derived mediators are known to be able to induce leukocyte activation and recruitment (Page and Pitchford 2013), as well as the release of substances that contribute to the remodelling and repair of tissues after injury, making them well placed to contribute to some of the important features of asthma (Fig. 1). Furthermore, it is now recognised that platelets from allergic asthma patients express both the high- and the low-affinity IgE receptors on their surface and that exposure to sensitising antigens can lead to the generation of inflammatory mediators, such as oxygen free radical species, 5-HT and RANTES (Joseph et al. 1983, 1997; Hasegawa et al. 2001; Klouche et al. 1997). Interestingly, activation of the high-affinity IgE receptor can cause platelets to undergo chemotaxis (Pitchford et al. 2008), confirming other observations that suggest that platelets behave like primitive leukocytes and demonstrate directional movement in response to certain stimuli (Czapiga et al. 2005; Kraemer et al. 2010), stimuli that tend to be distinct from those that elicit platelet aggregation (Abi-Younes et al. 2001; Kowalska et al. 2000; Clemetson et al. 2000).

Platelets have long been recognised to contain high concentrations of 5-hydroxytryptamine (5-HT or serotonin) in their dense granules, and several clinical observations

Table 1 Principal platelet-derived inflammatory mediators and their involvement in airway diseases

Mediator	Action
αvβ3	NF-κB activation and induction of inflammatory cell responses (cell adhesion and transmigration into the lung)
CD40L	TNF superfamily: antigen-presenting cell activation, B-cell responses, endothelial cell activation
P-selectin	Selectin: leukocyte adhesion, complement activation
IL-1β	Cytokine: acute phase response, leukocyte and endothelial activation
TXA2	Eicosanoid: T-cell differentiation, monocyte activation, smooth muscle proliferation
Nitric Oxide (NO)	Reactive oxygen species: anti-inflammatory and antithrombotic
ADP	Platelet, leukocyte, endothelial cell activation
TNFα	Modulate air wall remodelling
LTB4	Modulate leukocyte recruitment
CXCL-7/β-TG	Modulate leukocyte recruitment, bronchospasm
CCL5/RANTES	Monocyte adhesion on the endothelium and recruitment
CXCL4/PF-4	Chemokine: monocyte, neutrophil and T-cell recruitment, Th differentiation, bronchospasm
TGFβ	Cytokine: cell proliferation, T-cell differentiation, B-cell and macrophage phenotype regulation
PDGF	Growth factor: cell growth and differentiation, monocyte/macrophage differentiation
VWF	Platelet adhesion, PMN extravasation
SDF-1	Chemokine: T cell, monocyte and PMN chemotaxis
VEGF	Growth factor: angiogenesis, adhesion molecule expression
5-HT	Dendritic cell activation, bronchospasm

have shown altered levels of this vasoactive amine in patients with asthma (Maccia et al. 1977; Malmgren et al. 1982; Ring et al. 1980). Furthermore, some studies have shown a beneficial effect of the 5-HT antagonist ketanserin in asthma (Cazzola et al. 1990, 1992). Tryptophan hydroxylase (THP)-1 is a critical enzyme for the biosynthesis of 5-HT outside of the central nervous system (CNS), and it is of interest that Durk et al. have utilised mice genetically deficient in THP-1, as well as mast cell-deficient mice, to demonstrate that platelets, rather than mast cells, are the main source of 5-HT released during an allergic inflammatory response (Dürk et al. 2013). Unlike mice, human mast cells are not a major source of 5-HT and so it is of even more interest that Idzko and colleagues have found elevated 5-HT levels in BAL fluid following segmental bronchial allergen challenge in allergic asthmatics, as their results suggest that the 5-HT found in the lung likely comes from activated platelets (Dürk et al. 2013). These results provide further support to other clinical observations reporting platelet activation in patients with allergic asthma (Maccia et al. 1977; Kowal et al. 2006; Pitchford et al. 2003; Johansson et al. 2012). It is of particular interest that platelets have now been found extravascularly in a number of inflammatory situations (Feng et al. 1998), including in the lungs of experimental animals (Pitchford et al. 2008) and patients with asthma (Jeffery et al. 1989). It becomes intriguing to know whether the elevated 5-HT found in BAL of allergic asthmatics is derived from circulating platelets and/or from platelets that have undergone diapedesis into lung tissue. Either way the results presented by Durk et al. are of further interest as they have reported that allergic mice deficient in THP-1 or treated with an inhibitor of THP-1, PCPA, exhibit reduced leukocyte infiltration into the lung and inhibition of the bronchial hyperresponsiveness (BHR) that normally accompanies allergen challenge, suggesting that the platelet-derived 5-HT is playing a central role in allergic inflammation in the lung. Given that there are some encouraging early clinical observations in patients with asthma administered drugs either affecting 5-HT uptake (Lechin et al. 1998) or antagonising 5-HT2 receptors (Lima et al. 2007), it would seem timely to consider large trials of such agents in patients with allergic airways disease.

Studies in Experimental Animal Models: Leukocyte Recruitment

There is now a growing body of literature that suggests that platelets play a central role in both allergic (Pitchford et al. 2003, 2005) and non-allergic (Kornerup et al. 2010) leukocyte recruitment into the lung, as well as a critical role in other manifestations of allergic asthma such as airway remodelling (Pitchford et al. 2004). The mechanisms by which platelets influence these processes are discussed below and open up the possibility of identifying a number of potential new targets for the treatment of inflammatory diseases such as asthma and COPD.

Investigation of the mechanics of blood flow (haemorheology) has uncovered interesting phenomena that have fundamental implications related to how platelets are required to initiate the ‘adhesion cascade’ that results in leukocyte recruitment into tissues associated with inflammatory airway diseases. In laminar flow in blood vessels, a shearing motion occurs due to the influence of wall friction that results in a

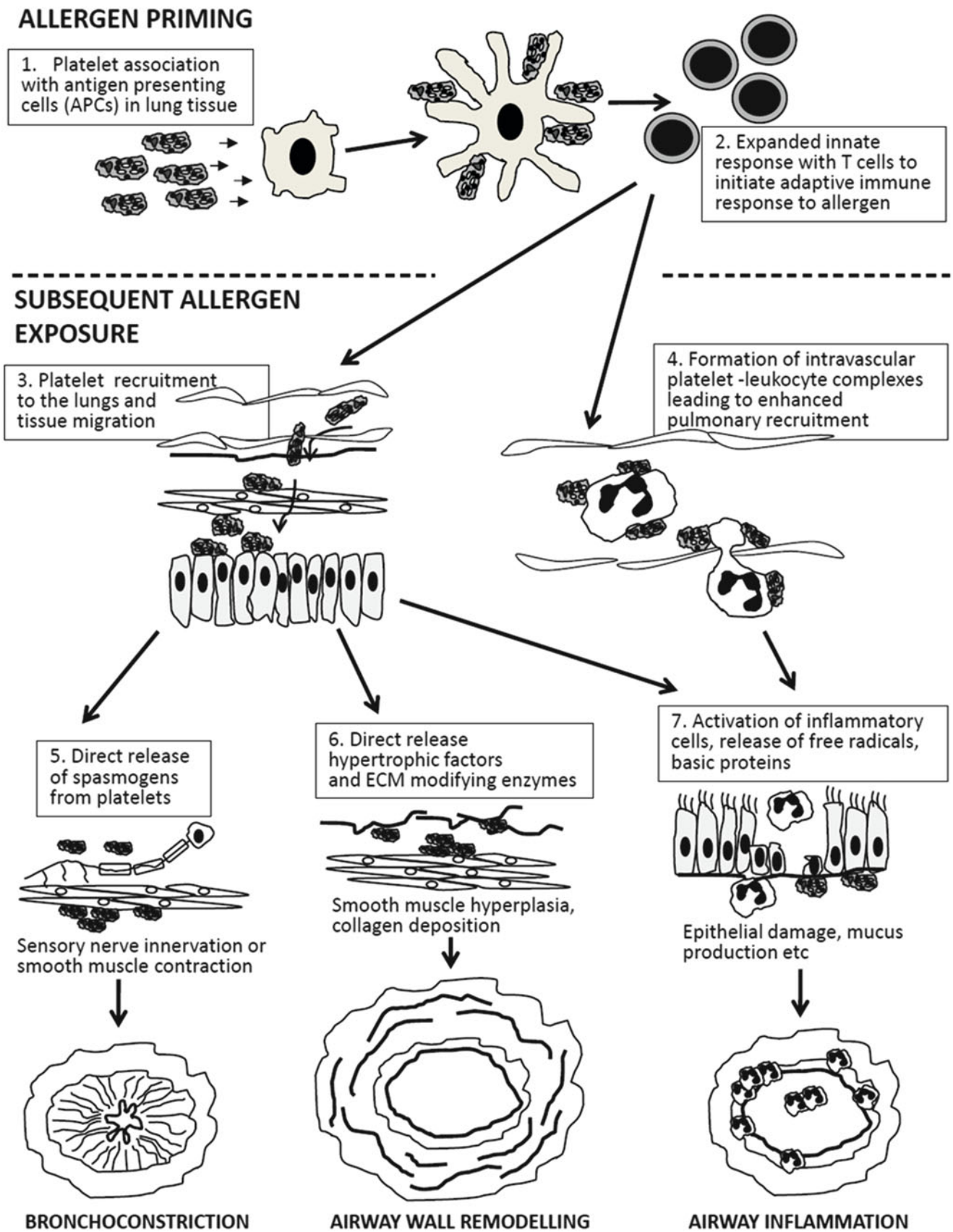

Fig. 1 There is evidence for platelet participation in both innate immune surveillance and the adaptive response to allergen and inflammatory events that occur after subsequent secondary exposure. This picture was originally published in Idzko, Pitchford, and Page J Allergy Clin Immunol (2015) 135: 1416-1423

parabolic velocity profile of the fluid (Hagan–Poiseuille Law); because blood is a non-Newtonian fluid, the viscosity of blood decreases with increasing shear rate and thus red blood cells actually aggregate to form reversible rouleaux under conditions of low shear and move inwardly (due to their relative deformability compared to platelets and leukocytes) into the inner part of the vessel (Goldsmith and Spain 1984a, b; Goldsmith et al. 1981, 1999). This phenomenon is known as the Fahraeus effect, the consequence of which is a redistribution of the blood elements, with the density of platelets and leukocytes increasing around the vessel periphery. The region of the vessel where the velocity gradient is highest is around the vessel wall; thus cells travelling at different velocities along adjacent streamlines have increased probability of cellular collisions. This peripheral zone therefore 'traps' leukocytes into an environment rich in platelets, greatly enhancing collisions between platelets and leukocytes (Goldsmith and Spain 1984a, b; Goldsmith et al. 1981, 1999) and leading to the tethering of platelets to leukocytes to form rosettes through P-selectin recognition steps, to the subsequent upregulation of integrin expression and to the firm adhesion to endothelium as platelet-bound leukocytes enter the capillary network. High-resolution video microscopy has revealed the existence of membrane tethers involving P-selectin/PSGL-1 bonds that regulate leukocyte rolling on platelets and P-selectin with changes in tether length and lifetime dependent on increasing shear force (Schmidtke and Diamond 2000). The biomechanics of the complex formation of activated platelets with leukocytes reveals that the high tensile strength of P-selectin/PSGL-1 interactions enables P-selectin-dependent tethering at high shear rates, whereas integrin activation may mediate platelet–leukocyte complex formation at low shear rates. Nevertheless, unstimulated leukocytes (which constitutively express PSGL-1/L-selectin) may also bind to activated platelets in an integrin-independent manner, suggesting that cell adhesion that is not purely selectin dependent may create platelet–leukocyte complexes (Xiao et al. 2006).

These events occur in the circulation of patients with asthma upon allergen challenge. Thus, circulating platelet–leukocyte complexes are found in allergic asthmatic patients after both spontaneous asthma attacks and after allergen challenge, in a biphasic manner (Gresele et al. 1993; Pitchford et al. 2003). The possible significance of these platelet–leukocyte complexes is to act as a 'priming' step for further leukocyte adhesion, since leukocytes attached to platelets display enhanced expression of the β1 integrin MAC-1 (Pitchford et al. 2003, 2005; Johansson et al. 2012). It is therefore of considerable interest that recent research has correlated platelet activation with eosinophil inflammation in patients with asthma (Benton et al. 2010). Furthermore, in patients with asthma, β1 integrin expression on eosinophils correlated with eosinophil-bound platelets expressing P-selectin and antigen challenge leads to the disappearance from the circulation of eosinophils bearing platelet P-selectin, presumably because these complexes are sequestered in the lungs (Johansson et al. 2012). The mechanistic significance of these platelet/eosinophil interactions is that this leads to increased eosinophil adhesion to the vascular endothelium via a platelet-P-selectin-dependent mechanism (Jawień et al. 2002; Ulfman et al. 2003).

The importance of platelets in pulmonary leukocyte recruitment after allergen challenge has now been extensively proven using in vivo models of platelet depletion. In fact platelet depletion, either via immunological or non-immunological methods, strongly reduces pulmonary eosinophil and lymphocyte recruitment in rabbits, guinea pigs and mice (Pitchford et al. 2003, 2005; Coyle et al. 1990; Lellouch-Tubiana et al. 1988) and the recruitment of effector T cells in murine models of contact hypersensitivity (Ludwig et al. 2004; Tamagawa-Mineoka et al. 2007). This process required intact platelets, since the reinfusion of lysed platelet products was insufficient to restore leukocyte recruitment, whereas the reinfusion of intact activated platelets expressing selectins on the cell surface restored leukocyte recruitment (Pitchford et al. 2005; Tamagawa-Mineoka et al. 2007). With similarities to allergic asthmatic patients undergoing allergen challenge, mice sensitised to allergen have circulating leukocytes attached to platelets displaying significantly increased CD11b (integrin αM) and VLA-4 (very late antigen-4) in comparison with leukocytes not attached to platelets (Pitchford et al. 2003, 2005). Contact with platelets therefore induces activation of leukocytes, thus enhancing the expression of integrins, presumably for firm adhesion. This has been confirmed by the experimental use of antibodies blocking P-selectin which results in the suppression of platelet–leukocyte complexes, integrin expression and subsequent tissue recruitment in models of asthma and chronic contact hypersensitivity (Pitchford et al. 2005; Ludwig et al. 2004; Tamagawa-Mineoka et al. 2007; Symon et al. 1999; Mayadas et al. 1993; Lukacs et al. 2002; De Sanctis et al. 1997; Broide et al. 1998). Indeed, the targeting of P-selectin or PSGL-1 as a novel therapeutic option is currently progressing in phase II clinical trials in patients with asthma (Bedard and Kaila 2010).

Nevertheless, many other studies also reveal that other mediators released, or expressed, by platelets can modulate leukocyte recruitment. Examples are the platelet-specific chemokines PF-4 (CXCL4), β-TG (CXCL-7) and RANTES (CCL5) and pleiotropic mediators such as leukotrienes, 5-HT and sphingolipids (Page and Pitchford 2013; Brandt et al. 2000; Duerschmied et al. 2013; Florey and Haskard 2009). Thus, whilst there is a requirement for platelets expressing adhesion molecules on their surface for leukocyte recruitment, this must also be sequential to the release or expression of other platelet-derived factors. However, it is not yet understood from a physiological perspective why

the process of leukocyte activation and adhesion to post-capillary venules is so inefficient and actually requires platelet activation. Perhaps the rapidity of platelet activation to danger signals combined with a higher surface area/volume ratio, allowing a critical density of adhesion molecules to be expressed compared to larger leukocytes, has resulted in an evolutionary requirement for platelets in the rheological processes that trigger the leukocyte adhesion cascade.

Pathophysiologic Observations: The Extravascular Presence of Platelets

Current perceptions concerning platelet function in inflammation are heavily influenced by the knowledge of the intravascular role of platelets in haemostasis and thrombosis. Thus, dogma suggests that the participation of platelets in inflammation is also confined to intravascular events and therefore that it is indirect and totally dependent on the ability of platelets to influence leukocyte recruitment. However, accumulating evidence details a novel function of platelets in being able to respond to chemotactic signals and migrate extravascularly through inflamed tissue (see Petito et al. 2017) (Fig. 2).

Platelets have been observed to undergo diapedesis in sections of the lungs obtained from patients with asthma and the lungs (and BAL fluid) from allergen-sensitised and allergen-exposed mice, rabbits and guinea pigs (Jeffery et al. 1989; Metzger et al. 1987; Pitchford et al. 2008; Lellouch-Tubiana et al. 1988; Beasley et al. 1989). Using quantitative histology, we have recently reported that the migration of platelets into lung tissue and the localisation of platelets around the airway wall in allergen-sensitised and allergen-exposed mice was an IgE-mediated process and that platelets from such mice could also undergo chemotaxis to the sensitising allergen in vitro (Pitchford et al. 2008). Interestingly, the migratory response of platelets in the lungs in vivo commenced before significant leukocyte recruitment, whilst at later time points, when leukocytes had also entered lung tissue, around 50 % of platelets were still not complexed to leukocytes (Pitchford et al. 2008). The rapidity of this response highlights that platelet activation by allergen is direct and independent of activation of other cells, like mast cells (Yoshida et al. 2002). It is interesting to note that evidence is accumulating of platelet migration into tissues in other inflammatory diseases, such as into the synovial fluid in patients with rheumatoid arthritis, and transmigration across the vascular wall after long periods of ischaemia (see Ware and Post 2017) (Boilard et al. 2010; Kraemer et al. 2010). The mechanisms controlling this platelet function have not been elucidated, but platelets express a number of different chemokine receptors (CCR1, CCR3, CCR4 and CXCR4) which are functional,

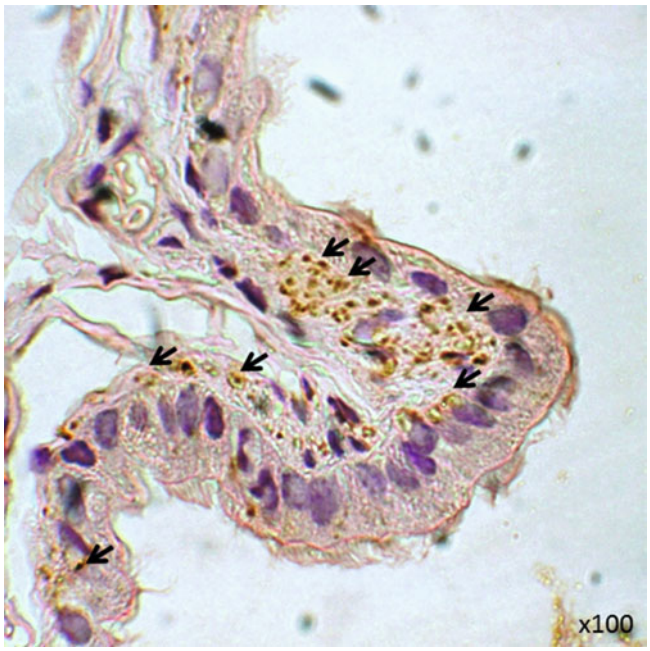

Fig. 2 Histological analysis of the lungs taken from ovalbumin (OVA)-sensitised mice after allergen inhalation. Section was stained for the platelet-specific antigen CD41 (integrin αIIb). Platelets (*brown*) can be observed throughout the airway wall. Image originally published in Page & Pitchford (2014) Clin Exp Allergy 44: 901–913

since the ligands SDF-1 α, MDC and TARC can activate platelets (Abi-Younes et al. 2001; Kowalska et al. 2000; Clemetson et al. 2000). Platelets have also been shown to undergo chemotaxis to f-MLP and SDF-1α and can therefore be considered as motile cells (Czapiga et al. 2005; Kraemer et al. 2010). Therefore, platelets can migrate through lung tissue and localise to specific resident cells/structures in a highly regulated process, as it is for leukocytes. Whilst the significance of platelet migration into lung tissue has not yet been fully characterised, platelets may directly influence the development of sensitisation towards allergen, BHR, bronchospasm, tissue damage and chronic inflammation leading to airway wall remodelling.

Studies in Experimental Animal Models: Involvement with Antigen-Presenting Cells and Contact with Allergen

Platelets express both the high-affinity (FcεRI) and low-affinity (CD23) receptors for IgE, as well as functional receptors for other immunoglobulins. Platelets can be activated by specific allergen via IgE-dependent processes, as revealed when platelets are taken from patients allergic to *Dermatophagoides pteronyssinus* (Der p1) and exposed to synthetic peptides derived from the allergen Der p1 ex vivo (Cardot et al. 1992). We have shown that platelets from

patients with asthma will undergo chemotaxis specifically towards the known allergen (rather than allergens to which individual patients are not allergic too) (Pitchford et al. 2008). The implications of direct platelet activation and motility by allergen are not yet known, but it is interesting to note that platelets activate dendritic cells (DCs) in the airways (Dürk et al. 2013). Both CD40 and CD40L have been identified on activated platelets and might be responsible for this cellular interaction (Semple et al. 2011; Henn et al. 1998). Platelet CD40L has been described in other situations as an important link between the innate and adaptive immune response to induce DC maturation, for example, the expression of CD80 and CD83, and immunoglobulin class switching (Elzey et al. 2003; Czapiga et al. 2004; Sprague et al. 2008). Platelet CD40L has recently been reported to be involved in the promotion of allergic airway inflammation by polarising Th2 responses after allergen exposure (Tian et al. 2015). Despite this evidence, it remains to be understood whether platelets are involved in the initial allergen sensitisation process per se.

Studies in Experimental Animal Models: Lung Function

The observation that platelets migrate into the lung tissue of patients with asthma, and into the lungs of allergen-challenged sensitised animals, opens the possibility that platelets may contribute directly to alterations in lung function in patients with asthma. For example, platelet depletion in allergen-sensitised rabbits and guinea pigs abolishes bronchoconstriction and anaphylaxis induced by inhaled spasmogens or allergens, respectively (Coyle et al. 1990; Lellouch-Tubiana et al. 1988). There is now some understanding of the pathways and platelet mediators involved in these processes from the observations on the effects of intravenous platelet agonists on bronchospasm and platelet accumulation in the lung (Arnoux et al. 1988; Yoshimi et al. 2001; Lellouch-Tubiana et al. 1988; Robertson and Page 1987). We have recently observed that platelet depletion inhibits bronchospasm induced by 'indirect spasmogens', such as capsaicin and bradykinin, whilst it does not inhibit direct-acting spasmogens, such as histamine and methacholine (Keir et al. 2015), suggesting that platelet-derived mediators contribute to airway obstruction under certain circumstances. Furthermore, the inhibition of the release of bronchoactive agents from platelets abrogated the resulting changes in airway obstruction, confirming that platelet-derived mediators might also contribute to airway tone (Arnoux et al. 1988; Yoshimi et al. 2001). Indeed, a direct participation of platelets in allergy, independent of leukocyte responses, was highlighted by the intradermal injection of supernatants from activated human platelets (but not leukocytes) inducing delayed, sustained inflammatory responses in the skin of patients with atopic dermatitis (Matsuda et al. 1997). These effects on tissue suggest that platelets are very capable of directly inducing sustained inflammation. Human platelets synthesise and release a number of bronchoactive mediators, for example, histamine, 5-HT, TXA2, adenosine and 12-hydroxyeicosatetraenoic acid (12-HETE), contain cytotoxic compounds within their granules and generate substances capable of inducing tissue damage, such as reactive oxygen species (ROS), cationic proteins (PCPs), platelet basic proteins (PBPs) and matrix metalloproteinases (Saxena et al. 1989; Knauer et al. 1984; Busti et al. 2010). Yet it is not understood how these mediators interact, and with what (e.g. sensory nerves, airway smooth muscle, epithelium), to elicit bronchospasm and BHR.

Studies in Experimental Animal Models: Chronic Inflammation and Lung Remodelling

One consequence of persistent, chronic inflammation is the alteration of tissue structure and function. In bronchial asthma, chronic inflammation contributes to changes in airway architecture referred to as 'airway remodelling'. The observation that platelets migrate into lung tissue of patients with asthma and in experimental animals (Jeffery et al. 1989; Metzger et al. 1987; Pitchford et al. 2008; Lellouch-Tubiana et al. 1988; Beasley et al. 1989) suggests that platelets may contribute directly to changes in the airway architecture by releasing factors that control the synthetic phenotype of the airway epithelium and of fibroblasts and airway smooth muscle cells. Indeed, in murine models of chronic allergic inflammation, the depletion of platelets led to a comprehensive suppression of lung remodelling (smooth muscle hyperplasia, subepithelial fibrosis, collagen deposition, epithelial hyperplasia), an effect that chronic treatment with glucocorticosteroids did not attain, suggesting that platelet involvement in tissue remodelling may in some instances be independent of leukocyte-associated inflammation (Pitchford et al. 2004). In patients with asthma, platelet activation was shown to persist for some time after the late asthmatic response had occurred (Kowal et al. 2006), even though the increases in circulating platelet–leukocyte aggregates returned to basal levels 24 h after allergen exposure (Pitchford et al. 2003), thus implicating platelets in chronic inflammatory events and airway remodelling.

Platelets are a rich reservoir of mitogens and enzymes and therefore may contribute to generate an environment that induces synthetic responses in structural airway cells. Platelet mitogens include PDGF, EGF (epidermal growth factor), TGF-β (transforming growth factor-β), VEGF (vascular endothelial growth factor) and the major product of arachidonic acid metabolism in platelets, TXA2, which all have proliferative activity on structural cells of the airways (Pitchford and Page 2002; Rendu and Bohard-Bohn 2002).

Platelet-derived enzymes include matrix metalloproteinases, β-hexosaminidases and heparanases that are released following allergen challenge in asthmatic patients and following ozone challenge in guinea pigs and may alter the composition of the extracellular matrix (Falcinelli et al. 2005; Kelly et al. 2000). Disruption of the composition and integrity of cell membranes by degradation of glycoproteins, glycolipids and glycosaminoglycans may also release membrane-bound growth factors for wound repair (McKenzie 2007). Recent evidence shows that platelet membranes are required to induce synthetic responses in airway smooth muscle cells (Svensson Holm et al. 2011), and this function was controlled by platelet 5-lipoxygenase, 12-lipoxygenase and the production of reactive oxygen species (Svensson Holm et al. 2008, 2014). These effects may be supplemented by the ability of platelets to influence the survival, recruitment, proliferation and differentiation of circulating stem cells involved in (inappropriate) tissue regeneration (Stellos et al. 2010). It can be surmised therefore that platelets may influence lung regeneration as well as inappropriate remodelling of the airways after injury, although the interplay between platelets and different structural cells is likely to be extremely complex and involve a plethora of mediators.

Platelets in Infectious Pulmonary Disease

- Platelets have a broad repertoire of receptor molecules that enable them to sense invading pathogens and infection-induced inflammation.
- Consequently, platelets exert direct antimicrobial mechanisms and initiate an intense crosstalk with other cells of the innate and adaptive immunity, including neutrophils, monocyte/macrophages, dendritic cells and B and T lymphocytes, favouring antimicrobial activity.

In mammalians, platelets have retained key structures and functions of immune effector cells which are integral to antimicrobial host defence (Morrell et al. 2014). In the bloodstream they act as quiescent 'sentinels' of tissue injury and microbial threat. Platelets exert activities that span from acute inflammation to adaptive immune responses that confer to them an important role as immune regulators during sepsis (Semple et al. 2011). Platelets store antimicrobial peptides, such as β-defensins, thrombocidins, PF-4 (CXCL4), RANTES (CCL5), connective tissue-activating peptide-3, platelet basic protein, thymosin β-4, fibrinopeptide B and fibrinopeptide A, and contribute to the innate immune system (Tang et al. 2002; Kraemer et al. 2011). Inflammatory lung diseases and other pulmonary disorders cause accumulation of platelets in the lungs, and tissue injury and systemic conditions such as sepsis can activate platelets in the circulation and sequester them in the pulmonary vascular bed (Fig. 3).

Pathophysiologic Observations

Platelets participate in inflammatory and immune responses using a variety of molecular mechanisms, including the release of chemokines (β-TG, PF4, ENA-78, Gro-α, RANTES, SDF1α and others), cytokines (HMBG1) and growth factors (PDGF, VEGF, EGF, FGF, TGFbeta, and so on), and the synthesis of lipid mediators, like thromboxane A2 and PAF, but contribute also to the maintenance of the endothelial barrier. Key differences exist between the characteristics of endothelium in the pulmonary circulation and the systemic vasculature. Notably, ICAM-1 and integrin $\alpha_v\beta_3$ are highly expressed by the pulmonary circulation as compared to other vascular beads (Panes et al. 1995; Singh et al. 2000). It is thus possible that platelet retention in the lungs is mediated by the constitutive expression of these two adhesion molecules by pulmonary endothelial cells. Activation of platelets, which is a requirement for their firm adhesion to endothelial cells, results in a conformational change

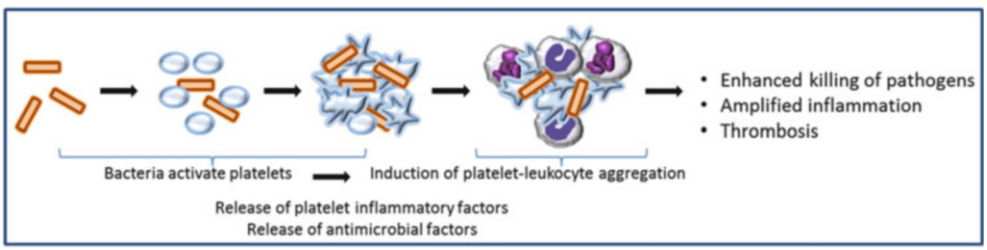

Fig. 3 Direct interaction of bacteria with platelets can lead to aggregation, release of antimicrobial factors and pro-inflammatory molecules and formation of platelet–leukocyte aggregates. Under some conditions, bacterial toxins of several classes can trigger these responses of human and murine platelets. Evidence suggests that these activities mediate immobilisation and killing of bacteria and other pathogens. In addition, however, each can contribute to local thrombosis and inflammation

in platelet $\alpha_{IIb}\beta_3$ that increases its affinity for fibronectin, which can then act as a bridge to endothelial cell $\alpha_v\beta_3$ in the low shear stress circulation of the pulmonary vascular bed (Goldenberg and Kuebler 2015). Moreover, platelets can induce inflammatory responses by expressing CD40L which is recognised by surface CD40 on endothelial cells which will then secrete adhesion molecules and TF (Freedman 2003). In addition, activated platelets display P-selectin which mediates their adhesion to PMN by binding to PSGL-1, favouring tethering and rolling on the endothelium, thereby guiding neutrophils to transmigrate (Weyrich et al. 2009) (Fig. 4).

Platelets express a ligand for triggering receptor expressed on myeloid cells (TREM)-1, a surface receptor that plays important roles in innate and adaptive immunity expressed by neutrophils and monocytes. Platelets bound to endothelium support the engagement of neutrophil TREM-1 and result in increased expression of pro-inflammatory chemokines and cytokines, thus amplifying the inflammatory response (Haselmayer et al. 2007). Platelets additionally function as a threshold switch for the release of neutrophil extracellular traps (NETs), web-like structures of DNA that can trap and kill microbes in the vasculature, by neutrophils (Brinkmann et al. 2004) (Rondina et al. 2017; Pryzdial et al. 2017; Foote et al. 2017). This occurs primarily in small vessels, like the liver sinusoids and pulmonary capillaries, where NETs, and particularly the histone components, are responsible for death of lung epithelial and endothelial cells, often with consequent tissue damage and organ dysfunction (Saffarzadeh et al. 2012).

Platelets also express Toll-like receptors (TLRs), a highly conserved family of pattern recognition receptors expressed from flies to mammals. TLRs bind pathogen-associated molecules that are broadly expressed by many infectious organisms. LPS is the most studied TLR ligand, and others include unmethylated cytosine guanine dinucleotide, double-stranded RNA and lipoproteins. Platelets express low levels of TLR2 (Aslam et al. 2006), TLR4 and TLR9 (Cognasse et al. 2005). In addition, additional TLRs and other secondary signalling molecules can become expressed following platelet activation. For example, high concentrations of thrombin induce the expression of TLR9 by platelets. TLR2 and TLR4 are functional in inflammatory responses such as sepsis, e.g. TLR4 triggers the expression of IL-6 and cyclooxygenase 2 (COX-2) in response to LPS (Scott and Owens 2008). LPS has been shown to induce thrombocytopenia and platelet accumulation in the lungs of wild-type but not TLR4-deficient mice. Moreover, LPS stimulation of platelet TLR4 induces platelet binding to and activation of adherent neutrophils. Indeed, stimulation of platelet TLR2 by synthetic bacterial lipopeptide directly activates the platelet's thrombotic and inflammatory response through the phosphoinositide 3-kinase (PI3-K) signalling pathway (Blair et al. 2009). In sepsis, neutrophils and

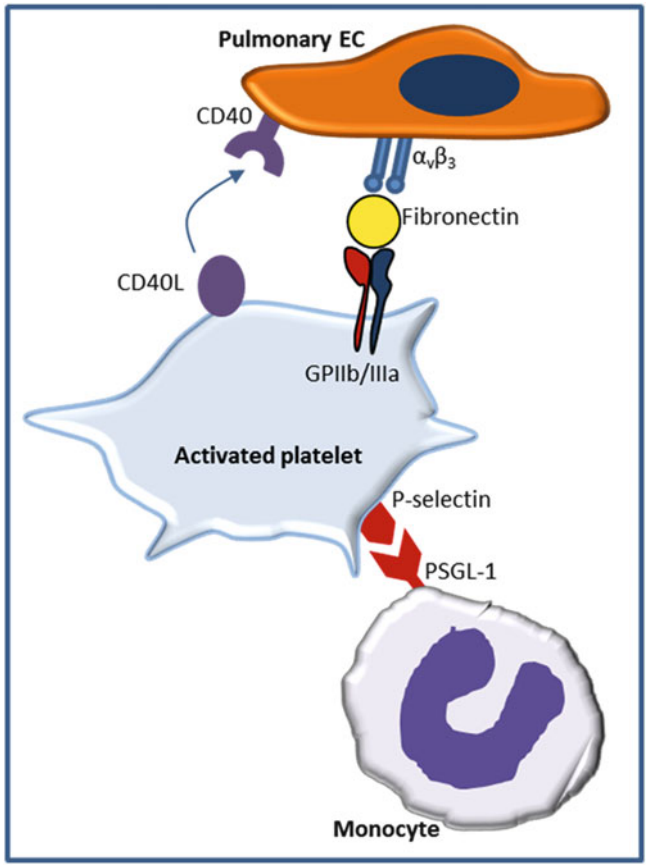

Fig. 4 Interactions between platelets and pulmonary ECs and monocytes in pulmonary infectious disease

platelets co-localise in several organs. Upon stimulation via TLR4, platelets induce neutrophil activation and the formation of NETs that display proteolytic activity, leading to the killing of microbes (Clark et al. 2007). Furthermore, platelets directly kill bacteria by releasing microbicidal peptides or by phagocyticing bacteria (Semple et al. 2011; Kraemer et al. 2011; Krijgsveld et al. 2000). Exposure of platelets to Gram-negative bacteria induces platelet aggregation, activation, P-selectin expression and formation of platelet/neutrophil aggregates via TLR2 (Beaulieu et al. 2011). Moreover, incubation of megakaryocytes with TLR2 leads to the generation of platelets with an enhanced pro-inflammatory gene and protein expression profile (Aslam et al. 2006).

In addition to their roles in innate immunity, platelets act as mediators between innate and adaptive immune response cells (Aslam et al. 2006; Elzey et al. 2003, 2005; Semple et al. 2011). Thus, platelets serve as early effectors of dendritic cells (DCs) activation in tissue injury through the release of CD40L and IL-1β and DCs are essential to link innate and adaptive immunity (Medzhitov and Janeway 2000; Banchereau and Steinman 1998). Platelet-mediated leukocyte adhesion to endothelium may also enhance lymphocyte trafficking during adaptive immunity and host

defence (Diacovo et al. 1996; von Hundelshausen and Weber 2007).

Studies in Experimental Animal Models

Platelets have a key role in immune surveillance by monitoring the surface of active macrophages (Wong et al. 2013). Platelets survey liver Kupffer cells through transient interactions via their GPIb with VWF constitutively expressed on Kupffer cells. When Kupffer cells capture blood-borne *S. aureus* or *Bacillus cereus*, they upregulate their VWF and the normally transient interaction with platelets becomes a sustained adhesion that actively encases the bacteria for eradication within the first minute of encounter. GPIb-deficient mice are significantly more susceptible to Kupffer cell damage and mortality in infections generated by the above-mentioned bacteria, ostensibly due to inefficient detection of Kupffer cell infection by platelets (Wong et al. 2013). Therefore, the targeting of infected macrophages by platelets seems to be an early and key host defence step to facilitate bacterial clearance and protection against disseminated infection (see Slaba and Kubes 2017).

The depletion of platelets in mice submitted to Klebsiella-induced pneumosepsis enhanced mortality in a way proportional to the extent of platelet depletion, by increasing the release of pro-inflammatory cytokines. Although Klebsiella sepsis did not directly enhance the expression of P-selectin on circulating platelets, the infection did increase the responsiveness of platelets to thrombin receptor stimulation (De Stoppelaar et al. 2014). In accordance, lipopolysaccharide (LPS) has been described to act as a platelet 'primer' (Gresele et al. 2008); in fact LPS induces platelet hypersensitivity to subthreshold concentrations of platelet agonists (Zhang et al. 2009).

During severe influenza A virus infection in mice, platelets massively infiltrated bronchoalveolar lavage fluid and were detected in ultrathin cryosections of the lungs staining positive for virus antihaemagglutinin, indicating that platelets actively incorporate influenza virus (Mazur et al. 2007).

Platelets in Cystic Fibrosis

- Platelets from cystic fibrosis (CF) patients have increased reactivity.
- A positive feedback loop of inflammation and platelet activation, which in turn lead to more inflammation and platelet activation, may exacerbate lung disease in patients with CF.

Cystic fibrosis (CF) is a multi-organ disease which affects the pancreas, the gastrointestinal tract, the male reproductive system and especially the respiratory tract. It is a lethal genetic disorder caused by a mutation in the gene, located in the long arm of chromosome 7, coding for the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel belonging to the superfamily of the ATP-binding cassette (ABC) proteins (Riordan et al. 1989). Chronic lung inflammation, due to an impairment of mucociliary clearance and to the consequent bacterial colonisation, represents the main cause of mortality and morbidity in CF patients.

Pathophysiologic Observations

CF patients have an increase in circulating activated platelets and platelet reactivity, as determined by enhanced monocyte–platelet aggregates, neutrophil/platelet aggregates and platelet surface P-selectin (Sturm et al. 2010; O'Sullivan et al. 2005). Moreover, elevated concentrations of sCD40L and TXA2 metabolites, which correlate with decreased lung function, further implicate platelets in the pathophysiology of this lethal disorder (Ciabattoni et al. 2000; Falco et al. 2004).

CFTR was found to be expressed by neutrophils and platelets, where it is localised in the cytosol, cell membranes and α-granules, and when blocked a markedly decreased thrombin-induced AKT phosphorylation and an upregulation of TRAP-stimulated p38MAPK phosphorylation were observed (Mattoscio et al. 2010). p38 MAPK activation has been associated with platelet secretion of pro-inflammatory mediators; thus the CFTR-dependent increase in p38 MAPK phosphorylation is consistent with a pro-inflammatory profile of CF platelets.

Another abnormality reported in CF patients is the reduction of plasma levels of vitamin E, which may increase fatty acid oxidation and thus the production of isoprostanes, which in turn activate platelets (Ciabattoni et al. 2000). Moreover, reduced nitric oxide (NO) generation was also observed in patients with CF and found to be a negative prognostic index associated with bacterial colonisation of the lung and sustained inflammation (Balfour-Lynn et al. 1996; Grasemann and Ratjen 2012). Endothelial dysfunction may also contribute to reduce NO generation in CF

(Poore et al. 2013), and the reduction of NO may contribute to platelet hyperreactivity. Platelets also produce NO upon eNOS activation that may act as a negative feedback mechanism to limit platelet activation and platelet recruitment to a growing thrombus (Cozzi et al. 2015; Momi et al. 2014; Freedman et al. 1997). Furthermore, the blockade of CFTR blunted NO generation by platelets suggesting this as another mechanism of the platelet hyperfunction in CF (Mattoscio et al. 2010).

Platelet 12-lipoxygenase is a key enzyme in the biosynthesis of lipoxins (Romano et al. 1993), arachidonic acid (AA) metabolites with potent anti-inflammatory properties. Lipoxin formation occurs in vivo during platelet–PMN interactions (Gangemi et al. 2003) and represents a main stop signal of inflammation (Serhan et al. 2008). Of interest, CF patients display a defect in the biosynthesis of lipoxin A4 (Karp et al. 2004), whereby mutated CFTR downregulates lipoxin formation by selective inhibition of platelet 12-lipoxygenase activity.

Patients with CF have increased plasma levels of ATP, due to the dysfunction of CFTR which acts as an ATP transporter, as well as a chloride channel, that can contribute to platelet activation (Lader et al. 2000). ATP may in fact act as a platelet agonist in vivo under special circumstances, like under high shear stress conditions, and may thus also contribute to the hyperreactivity of CF platelets (Birk et al. 2002).

Altogether, these data suggest an involvement of platelets in the sustained inflammatory response of CF originating from increased pro-inflammatory platelet-activating mediators and reduced counter-regulatory signals (Mattoscio et al. 2010; O'Sullivan et al. 2005).

Patients with CF show thrombocytosis, increased expression of P-selectin on circulating platelets (O'Sullivan et al. 2005), increased platelet reactivity to ADP and TRAP (Stead et al. 1987) and increased release of platelet-derived mediators (e.g. TNFα, CD40L, LTB4 and interleukins) (Shoki et al. 2013). Moreover, patients with CF display increased circulating platelet–leukocyte and platelet–monocyte complexes (O'Sullivan et al. 2005) and enhanced NET formation in airway fluids correlating with impaired obstructive lung function. These phenomena are blocked by the intra-airway delivery of small-molecule antagonists of CXCR2, with inhibition of NET formation and improved lung function (Marcos et al. 2010). In CF, increased platelet number correlated with decreased PaO2 (Gross and Luckey 1969), increased urinary thromboxane metabolite excretion with decreased FEV1 (Davì et al. 1995; Ciabattoni et al. 2000) and increased plasma sCD40L with decreased pulmonary function (Falco et al. 2004). Taken together these studies suggest a link between platelet activation and progressive impairment of lung function in CF. Whether platelet abnormalities are the cause or consequence of lung inflammation in CF is unclear. However, platelet dysfunction in CF could be the result of platelet abnormalities intrinsic to CF, of the accumulation of plasma factors unique to CF and/or of generalised inflammation.

Studies in Experimental Animal Models

Mice carrying the F508del mutation of CFTR developed more severe thrombocytopenia, higher levels of plasma TxB2 and PAF levels and enhanced neutrophilic lung infiltration when challenged with LPS relative to wild-type controls (Zhao et al. 2013). Blockade of PSGL-1 (P-selectin glycoprotein ligand-1) or of P-selectin, PAF-receptor antagonism or correction of mutated CFTR trafficking by KM11060 (an analogue of sildenafil, which restores a function of the F508del-mutated CFTR chloride channel) all significantly increased plasma lipoxin A4 levels, whilst the depletion of platelets significantly decreased plasma lipoxin A4 and enhanced LPS-induced lung inflammation (Wu et al. 2014).

Therefore, a growing body of evidence suggests that platelets play a very important role in CF and along with evidence of platelet involvement in other inflammatory lung diseases suggests that they may represent a potential therapeutic target in CF. However, much work remains to be done before anti-platelet therapy can be recommended for patients with CF.

Platelets in Chronic Obstructive Pulmonary Disease (COPD)

Platelet activation represents a mechanism that may contribute to the increased cardiovascular risk associated with stable COPD and with exacerbations.

Chronic obstructive pulmonary disease (COPD) is a respiratory disorder characterised by progressive lung destruction with airflow limitation associated with an airway and systemic inflammatory response (Rabe et al. 2007). Low-grade, chronic systemic inflammation in COPD is frequently complicated by acute atherothrombotic events, independent of the smoking history or other cardiovascular risk factors (Malerba et al. 2013), and coronary artery disease is one of the leading causes of death in COPD (Hansell et al. 2003). Moreover, COPD patients have an increased risk of ischaemic stroke (Truelsen et al. 2001) and venous thromboembolism, particularly during acute exacerbations (Tillie-Leblond et al. 2006). Although the mechanisms responsible for the association between COPD and atherothrombosis are still largely unknown, platelet activation is one of the proven links.

Pathophysiologic Observations

A link between hypoxia and platelet activation has been suggested since hypoxia facilitates the activation of cyclooxygenase-1 and thus thromboxane formation (Ponicke et al. 1987). More recently, a significantly enhanced urinary 11-dehydro TxB2 excretion, the urinary metabolite of platelet-released TxA2, was reported in patients with COPD, irrespective of the smoking status, that inversely correlated with arterial oxygen tension (Davì et al. 1997). Increased in vivo platelet activation in hypoxaemic COPD patients with secondary pulmonary hypertension was also detected by a significant increase in circulating platelet aggregates and plasma β-TG (Cordova et al. 1985), suggesting that activation of platelets in the pulmonary blood vessels may be associated with the induction of pulmonary hypertension.

Moreover, an elevation in arginase activity in platelets, which is associated with a reduction of NO production, has been described in COPD patients, and this may also contribute to enhanced platelet reactivity (Guzman-Grenfell et al. 2011). Increased levels of circulating soluble P-selectin, most likely of platelet origin, have also been observed in COPD patients (Ferroni et al. 2000), and platelet P-selectin is crucial for the formation of platelet–leukocyte aggregates (Totani et al. 2016). It is therefore conceivable that neutrophil recruitment to the lungs, a feature of COPD patients, is favoured by interactions with activated platelets. Platelet–leukocyte interactions mediate the process of NETosis (see Evangelista et al. 2017) and NETs are a major contributor to chronic inflammation and lung tissue damage in COPD (Grabcanovic-Musija et al. 2015). NETs were found in COPD patients and are associated with other markers of activated innate immune response, including the expression of the pro-inflammatory cytokines IL-1β and CXCL8 and of the inflammasome component NLRP3. Consequently NETs may contribute, through a positive feedback and the stimulation of neutrophilic chemokines and cytokines, to the persistent airway neutrophilia observed in COPD (Wright et al. 2016). Further research is required to elucidate the participation of platelets in NETosis and the exact role that NETs are playing in COPD. Furthermore, the use of anti-platelet therapy in patients has been shown to improve survival in patients with oxygen-dependent COPD (Ekstrom et al. 2013).

Platelets in Acute Lung Injury and Acute Respiratory Distress Syndrome

- Platelet/neutrophil interactions may be major pathogenic contributors in ALI.
- The interaction between platelets and neutrophils is mediated by platelet P-selectin and neutrophil PSGL-1.
- Platelets contribute to NET formation that involves the expulsion of nuclear material embedded with histones, by neutrophils in ALI/ARDS. Histones were found in the bronchoalveolar lavage fluid in ARDS; in a transfusion-related acute lung injury model in mice, depletion of platelets was protective.

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are life-threatening diseases occurring in critically ill patients. They are manifestations of an inflammatory response of the lung to direct and indirect insults and are characterised by severe hypoxaemia, hypercapnia, diffuse inflammatory lung infiltration and impairment of pulmonary compliance (Ragaller and Richter 2010). In particular, ALI is defined as an acute lung disease with bilateral pulmonary infiltrates in a chest radiogram consistent with the presence of oedema, and no evidence of pulmonary hypertension (Pepe et al. 1982). ALI is an important complication of sepsis.

Pathophysiologic Observations

Growing evidence suggests that platelets play a role in the pathogenesis of ALI (Zarbock et al. 2006). Intrapulmonary triggers, like acid aspiration and pneumonia, and systemic inflammatory stimuli activate the lung microvascular endothelium with upregulation of adhesion molecules, including ICAM-1 (Basit et al. 2006), release and presentation of chemokines (Belperio et al. 2006) and increase of lipid mediators (Zarbock et al. 2006; Kiefmann et al. 2004). During endotoxaemia platelets roll on and adhere to pulmonary capillary endothelial cells, as shown by intravital and electron microscopy (Kiefmann et al. 2006; Zarbock and Ley 2009), and release chemokines and lipid mediators that activate leukocytes and endothelial cells (Zarbock et al. 2006) (Fig. 5). Platelet microparticles (PMPs), small (50 nm to 1 μm) circulating cell-derived vesicles that break off from intact platelets upon activation and contain a variety of enzymes and proteins as well as mRNAs (McVey et al. 2012) (see Cointe et al. 2017), may play a role in ALI by promoting the inflammatory response of the lungs through the activation of neutrophil cell surface proteins (e.g. IL-8, MAC-1, PECAM-1, etc.) (Reutershan and Ley 2004; Bastarache et al. 2009; Shang and Yao 2014) and the production of cytokines and inflammatory mediators by endothelial cells (Eickmeier et al. 2013). A recent study showed that PMPs carry membrane-bound sCD40L, promote PMN-mediated HUVEC damage and may affect the

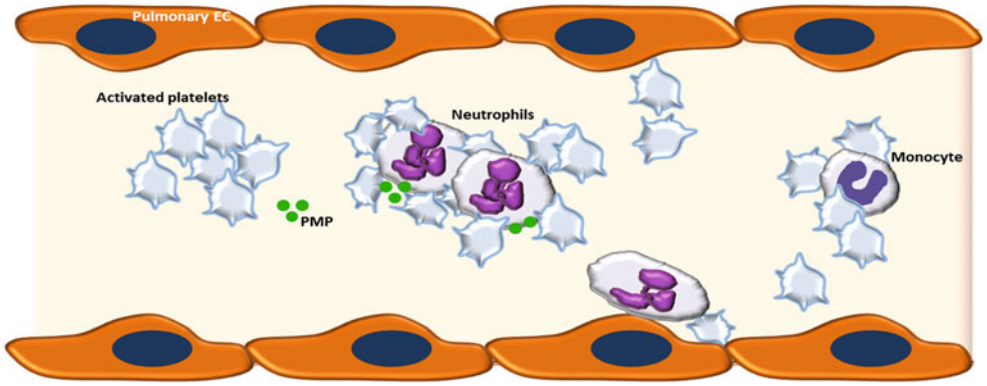

Fig. 5 Platelet–platelet, platelet–endothelial, platelet–PMN and platelet–endothelial cell–PMN interactions occur in pulmonary arterioles, capillaries and venules. These events can mediate acute lung injury. Different molecular interactions mediate adhesion of activated platelets to PMNs including P-selectin/PSGL-1, integrin MAC-1/integrin αIIbβ3 or ICAM-2, integrin MAC-1/GPIbα or JAM-3; ICAM-2/αLβ2 (LFA-1). Platelets also interact with monocytes under these conditions

development of ALI (Xie et al. 2015). Moreover, PMPs from LPS-stimulated platelets induce VCAM-1 production by cultured human endothelial cells (Levy and Serhan 2014).

Neutrophils and platelets are both key players in the pathophysiology of ALI. Thus, in a transfusion-related acute lung injury model in mice, depletion of either neutrophils or platelets was protective (Looney et al. 2009).

NETs are the product of a neutrophil death process that involves the expulsion of nuclear material embedded with histones, proteases, antimicrobial proteins and peptides. Histones have been found in the BAL fluid in acute respiratory distress syndrome (ARDS) (Bosmann et al. 2013). Platelets contribute to NET formation by neutrophils (see Slaba and Kubes 2017). Using an in vitro flow chamber system, it was shown that platelet-bound TLR4 detected TLR4 ligands in blood, activated neutrophils and facilitated the formation of NETs in the bloodstream. Moreover, using in vivo imaging in mice, it appeared that the lung capillaries where platelets bound neutrophils were hot spots for bacterial trapping and that the removal of platelets impaired bacterial clearance (Clark et al. 2007). Interestingly LPS, even at high concentrations, is not able to induce the formation of NETs in the absence of platelets, suggesting that platelets are not just facilitators in this process but essential mediators. On the other hand, the histone/DNA complexes of NETs activate platelets that in turn further promote NETosis, thrombosis and coagulation (Semeraro et al. 2011). Finally, extracellular histones, known to act as strong drivers of inflammation and tissue damage, activate TLRs resulting in pro-inflammatory cytokine production contributing to ALI development (Ward and Grailer 2014). All these observations point to an involvement of platelets in ARDS. One SNP within the LRRC16A gene (rs7766874), associated with variations in the platelet count, was involved in the pathophysiology of ARDS in a large series of 629 ARDS cases and 1,026 at-risk control subjects. However, it remained unclear whether the reduced platelet counts in patients with the LRRC16A SNP affected ARDS risk by a reduced platelet production, increased platelet activation/consumption or platelet sequestration in lung tissues (Wei et al. 2015).

Finally, another evidence has demonstrated that activated coagulation and impaired fibrinolysis are associated with ALI. During ALI, the coagulation system is activated, producing fibrin deposition in the lung (Idell et al. 1989) (Fig. 6). The newly produced thrombin, on the one hand, activates protease-activated receptors (PARs) on endothelial cells, inducing an inflammatory response with upregulation of cytokines as well as thrombin formation, whilst, on the other hand, it also activates platelets by binding to platelet PAR1 and 4 (Coughlin 2005). Thrombin also induces the conversion of fibrinogen to fibrin that, together with activated platelets, can induce the formation of microvascular thrombosis.

Studies in Animal Models

Inhibition of platelet/neutrophil aggregate formation resulted in reduced neutrophil recruitment, increased survival and lower levels of hypoxia in LPS-induced ALI in mice (Zarbock et al. 2006). In a ventilator-induced lung injury model in rats, increased levels of VWF were found on freshly isolated lung endothelial cells. The increased expression of VWF was inhibited by platelet removal from the lung perfusion and by a P-selectin-blocking antibody, and it was also drastically reduced in the lungs of mice perfused with blood from P-selectin knockout animals. These findings indicate that in ventilation-induced stress, platelets transfer VWF to endothelial cells and that platelet P-selectin plays a critical role in this

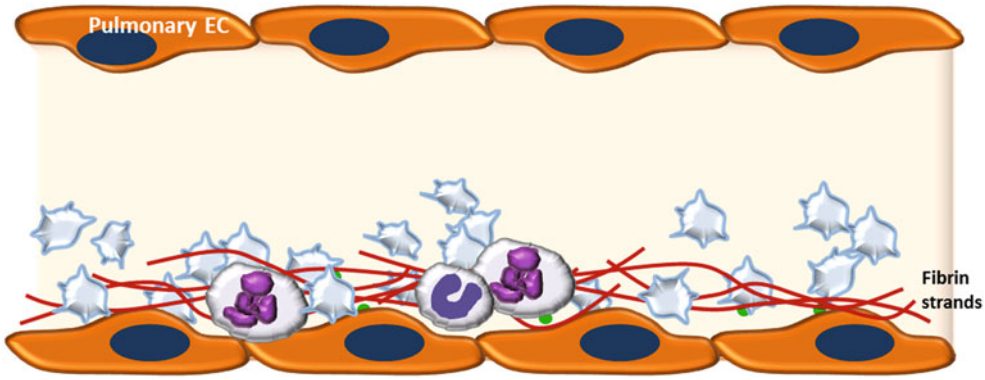

Fig. 6 Platelets and PMNs accumulate in macro- and microvessels in the lungs of patients with ALI/ARDS. Sequestration of platelets and PMNs is frequently associated with fibrin deposition and endothelial injury

transfer (Yiming et al. 2008). These data imply that during mechanical ventilation-induced lung injury, platelets deliver leukocyte-binding proteins to endothelial cells promoting leukocyte recruitment, thus playing a key role in generating a pro-inflammatory milieu.

Platelet depletion markedly reduced lung neutrophil infiltration in a murine model of lung injury induced by aerosolised LPS (Grommes et al. 2012). In the same model, antagonism of the CCL5-CXCL4 interaction reduced lung oedema, neutrophil infiltration and tissue damage, suggesting an important and causative role for platelet-derived chemokines in the development of lung injury (Grommes et al. 2012).

Moreover, in a mouse model of transfusion-associated lung injury (TRALI), platelet inhibition by aspirin or by a glycoprotein IIb/IIIa inhibitor reduced the degree of lung injury and the formation of NETs (Caudrillier et al. 2012). Therefore, platelet inhibition could reduce lung injury in ARDS by reducing the formation of NETs.

Take-Home Messages

A role for platelets in lung disorders has been revealed by studies in experimental animal models and in patients with different forms of acute and chronic lung injury with an inflammatory component, with platelets being critical for leukocyte recruitment, tissue damage and lung dysfunction. The complex web of cellular interactions that contribute to lung tissue damage, increasingly unravelled, includes now the humble platelet, and the improved understanding of the central pathophysiologic role of this cell may hold the key to improved treatments for these common conditions. It is becoming clear that the ability of platelets to act as inflammatory cells involves activation and signalling pathways distinct from those involved in haemostasis and thrombosis. This may provide new exciting opportunities for identifying new drug targets for the treatment of inflammatory lung disorders.

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Hemostatic and Non-hemostatic Functions of Platelets in Patients with Liver Disease

Ton Lisman

Abstract

Chronic and acute liver diseases may be accompanied by substantial changes in primary hemostasis. Thrombocytopenia develops as a consequence of defective platelet production, increased consumption, and/or increased splenic sequestration. In addition, ill-defined platelet function defects may further impair primary hemostasis. However, defects in platelet number and function appear (partly) compensated for by increased levels of von Willebrand factor (VWF) and decreased circulating ADAMTS13. Consequently, patients with liver diseases may have a relatively well-functioning primary hemostatic system, which does not necessarily necessitate prophylactic transfusion of platelet concentrates, for example, prior to invasive procedures. Thrombopoietin receptor agonists are able to increase the platelet count, but normalization of the platelet count in patients with cirrhosis may lead to a thrombotic risk due to the high plasma levels of von Willebrand factor. The platelet count is a reasonable diagnostic and prognostic tool in patients with liver diseases. Platelets also have functions unrelated to cessation of bleeding in the context of liver disease. By incompletely understood mechanisms, platelets contribute to regeneration of the liver, for example, following a partial hepatectomy. In animal models of chronic or acute liver failure, platelets have been shown to exert both detrimental and beneficial effects. Whether platelets can both stimulate and inhibit progression of liver disease depending on the exact clinical context requires further study.

Introduction

Patients with liver diseases may acquire complex alterations in their hemostatic system including thrombocytopenia and platelet function defects, decreased plasma levels of coagulation factors, natural inhibitors of coagulation, and fibrinolytic factors (Lisman et al. 2002). In addition increased plasma levels of hemostatic proteins including von Willebrand factor, factor VIII, tissue-type plasminogen activator, and plasminogen activator inhibitor type 1 are frequently found. The causes for these complex hemostatic changes are multifactorial and include a decreased synthetic capacity of the liver, consumption of coagulation factors by disseminated or localized activation of coagulation, chronic endothelial cell activation, and portal hypertension-associated splenomegaly (Lisman and Porte 2010). Historically, patients with liver diseases were thought to have a bleeding disorder related to hemostatic failure. The notion of a bleeding tendency in patients with liver disease was supported by abnormalities in routine diagnostic tests of hemostasis (such as the platelet count and the prothrombin time) in a patient population characterized by frequent bleeding episodes and substantial blood loss during major surgery such as liver transplantation (Lewis et al. 1987). Extensive laboratory studies combined with careful clinical observations have substantially altered the concepts on

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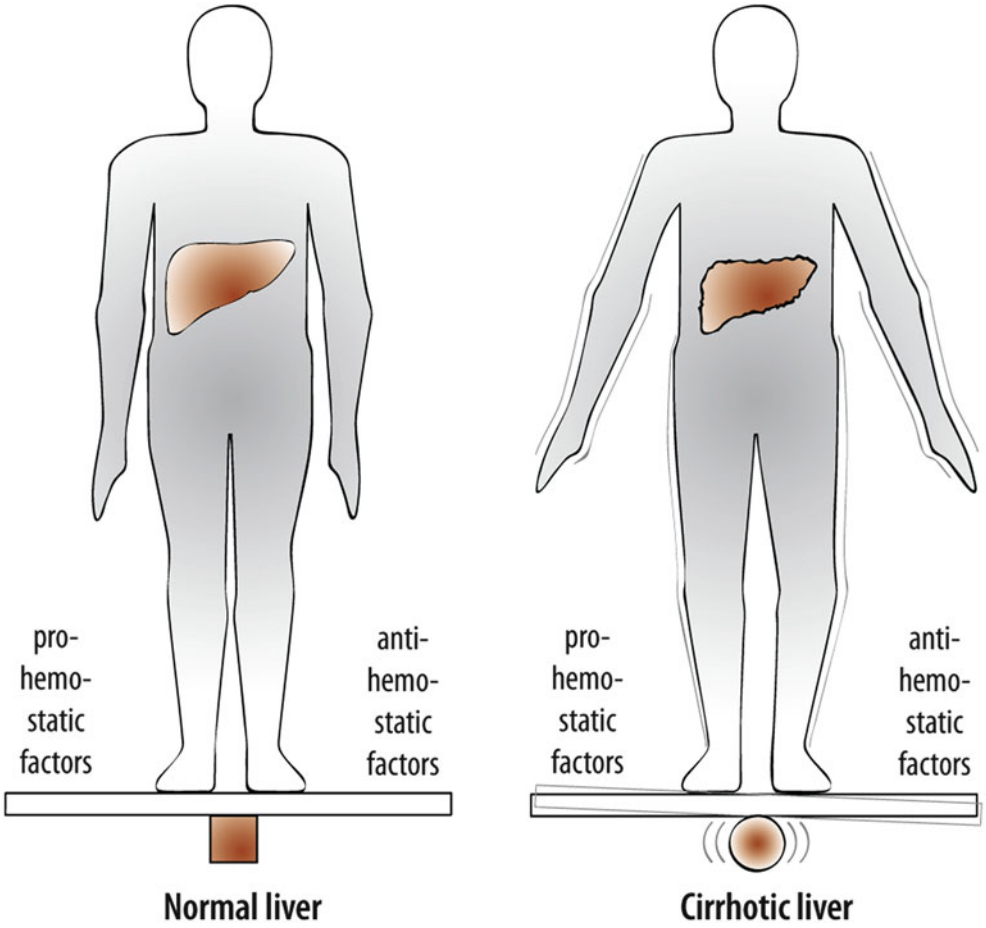

Fig. 1 The hemostatic balance in patients with liver disease as compared to that of healthy individuals. This cartoon depicts the stable hemostatic balance in healthy individuals and shows that although the hemostatic system in patients with liver disease is (re)balanced, the balance is fragile and may easily tip to either a hypo- or hypercoagulable status. Reprinted with permission from Lisman et al. (2013)

consequences of hemostatic disturbances in patients with liver diseases (Lisman and Porte 2010; Tripodi and Mannucci 2011; Northup and Caldwell 2013). The current belief is that the hemostatic balance in patients with liver diseases is relatively well preserved due to a concomitant decline in both pro- and antihemostatic pathways (Fig. 1). Clinical evidence for a "hemostatic rebalance" in liver diseases include developments in liver transplant surgery in which it has become clear that a proportion of patients can undergo this lengthy and invasive procedure without the requirements for any blood product transfusion (Massicotte et al. 2012), even in the presence of major laboratory abnormalities of hemostasis (Massicotte et al. 2008). In addition, it has become widely accepted that variceal bleeding, the prime bleeding complication of patients with chronic liver failure (i.e., cirrhosis), is a result of portal hypertension and local vascular abnormalities rather than a consequence of hemostatic failure (Garcia-Tsao and Bosch 2010). The hemostatic balance of patients with liver diseases, however, appears much more fragile compared to the hemostatic balance in individuals with competent liver function. This fragile hemostatic balance explains why patients with liver diseases may experience both hemostasis-related bleeding and thrombotic complications (Lisman et al. 2010).

This chapter will detail the abnormalities in primary hemostasis in patients with liver diseases and will provide guidance for clinical management of thrombocytopenia and platelet function defects in these patients. In addition, the prognostic value of the platelet count in chronic and acute liver diseases will be outlined. Finally, functions of platelets in liver diseases beyond cessation of bleeding will be discussed. These functions include stimulation of liver regeneration and roles in modulation of liver damage.

Part A: Alterations in Platelet Number and Function as a Consequence of Liver Disease

Thrombocytopenia of Liver Disease

Both cirrhosis and acute liver failure are frequently accompanied by thrombocytopenia. In more advanced cirrhosis, the majority of patients (50–75 %) have some degree of thrombocytopenia (a platelet count <150,000/μl), but

severe thrombocytopenia (<50,000/μl) is relatively rare at 1–11 % (Boyer and Habib 2015). The extent of thrombocytopenia increases with the severity of disease. Thrombocytopenia also frequently accompanies acute liver failure, although the proportion of patients with thrombocytopenia is lower and the extent of the platelet count reduction is milder in patients with acute liver failure compared to patients with cirrhosis, while the plasma levels of coagulation factors are much lower in acute liver failure compared to cirrhosis. In an analysis of 1598 patients from the US Acute Liver Failure Study Group, patients had a median platelet count of ~130,000 platelets/μl on admission, with 59 % of patients with a platelet count below 150,000/μl, 34 % of patients with a platelet count below 100,000/μl, and 9 % of patients with a platelet count below 50,000/μl (Lisman and Stravitz 2015). Platelet counts decline further after admission for ALF, reaching a nadir at day 4–5.

The thrombocytopenia of cirrhosis is multifactorial and may include defective synthesis of thrombopoietin (TPO) by the diseased liver (Peck-Radosavljevic et al. 2000), splenomegaly related to portal hypertension (Aster 1965), consumption as a result of disseminated or localized activation (Bakker et al. 1992; Kondo et al. 2013; Ikura et al. 2013), increased platelet destruction by autoantibodies directed to platelets (Kajihara et al. 2003), and direct bone marrow suppression by, for example, alcohol or the hepatitis C virus (Levine et al. 1986; Garcia-Suarez et al. 2000). Importantly, treatment of hepatitis C with interferon-based regimens causes a further decrease in platelet count, frequently necessitating dose adjustments or administration of thrombopoiesis-stimulating agents (Afdhal et al. 2008). The new generation antiviral agents do not appear to have this disadvantage (Pawlotsky et al. 2015). Although the liver is an important site of synthesis of TPO, decreased TPO synthesis does not appear to explain the thrombocytopenia of acute liver failure. In fact, TPO levels are normal-to-increased in most patients with acute liver failure, and levels further increase during the course of the disease, despite decreasing platelet counts (Schiodt et al. 2003). These observations are in contrast to the low plasma TPO levels in patients with cirrhosis (Peck-Radosavljevic et al. 2000). Whether TPO-mediated platelet production is intact in patients with acute liver failure, or whether thrombopoiesis is disturbed by mechanisms other than TPO deficiency, is unknown. Recent data suggest that platelet activation/consumption is an important cause for the thrombocytopenia of acute liver failure (Stravitz et al. 2016). In an analysis of platelet count over time in patients within the acute liver failure study group from the United States, the drop in platelet count over time increased in proportion to the extent of the systemic inflammatory response syndrome which is common in these patients. In addition, the drop in platelet count is accompanied by an increase in platelet-derived microparticles (Stravitz et al. 2013b, 2016). A similar mechanism has also been proposed to contribute to the thrombocytopenia of cirrhosis, but the presence of elevated levels of platelet microparticles in cirrhosis is controversial (Rautou et al. 2013; Tapper et al. 2013).

Platelet Function Defects

Already in the 1960s, it was shown that platelets from patients with cirrhosis have defective in vitro aggregability (Thomas et al. 1967). The decreased capacity to activate platelets from patients with cirrhosis in vitro has been confirmed by many laboratories (Desai et al. 1989; Ingeberg et al. 1985; Laffi et al. 1987; Alkozai et al. 2015). In addition, it has been shown that many patients with cirrhosis have a prolonged bleeding time that correlates poorly with the platelet count, which is indicative of a platelet function defect (Blake et al. 1990). Platelet function defects in cirrhosis have been ascribed to both intrinsic and plasma-derived factors. The intrinsic platelet defects include an acquired storage pool deficiency (Laffi et al. 1992), defective transmembrane signaling (Laffi et al. 1993) with defective synthesis of arachidonic acid (Laffi et al. 1987), and upregulation of cyclic AMP and GMP (Laffi et al. 1993) possibly as a result of an increased production of nitric oxide and prostacyclin in vivo (Cahill et al. 2001). Plasma-derived factors that inhibit platelet function in cirrhosis include abnormal high-density lipoprotein and bile salts (Desai et al. 1989; Baele et al. 1980). Platelet function in patients with acute liver failure has been poorly studied, but decreased platelet function has been shown in older studies (Rubin et al. 1977; Weston et al. 1977)

Hemostatic Status of Primary Hemostasis in Liver Diseases

Although platelet aggregation tests and other static in vitro tests of platelet function clearly suggest platelet hypofunction in patients with cirrhosis, there is no consensus that the thrombocytopenia and platelet function defects in cirrhosis translate to a defective primary hemostatic function. First, there is in vivo evidence of platelet hyperactivity including increased urinary excretion of the thromboxane A2 metabolite 11-dehydro-thromboxane B2 and increased plasma levels of platelet activation markers such as soluble P-selectin or platelet factor 4 (Davi et al. 1998; Panasiuk et al. 2001). Second, there is evidence for in vivo platelet activation including increased P-selectin expression on resting platelets and increased numbers of platelet-monocyte aggregates (Panasiuk et al. 2001; Sayed et al. 2010). This enhanced in vivo platelet activation may be related to

continuous endothelial activation. Alternatively, platelets may be activated during infection or as a result of systemic low-grade inflammation. Third, flow-based assays of platelet function including the PFA-100 and flow-based adhesion and aggregation studies used in research settings have suggested that platelet defects are explained by anemia and thrombocytopenia, but not by platelet function defects (Escolar et al. 1999; Lisman et al. 2006a). Finally, it has been proposed that highly elevated levels of von Willebrand factor (VWF) found in liver diseases compensate for the low platelet count in patients with liver disease (Lisman et al. 2006b). VWF levels are substantially elevated in both chronic (Lisman et al. 2006b) and acute (Hugenholtz et al. 2013) liver disease which is likely related to chronic endothelial cell activation, possibly in combination with decreased VWF clearance by the diseased liver. In addition, VWF synthesis may be increased, as liver disease is associated with induction of VWF expression in the liver (Hollestelle et al. 2004). Alternatively, VWF synthesis may be increased due to the substantially enhanced endothelial surface in patients with cirrhosis as a consequence of extensive collateral formation. VWF functionality is partially impaired, possibly due to proteolysis by proteases such as plasmin or elastase (Federici et al. 1993). Nevertheless, in flow-based in vitro assays, the quantitative increase by far overrules the qualitative defects resulting in a substantially increased adhesion of platelets to thrombogenic surfaces in the presence of cirrhotic plasma containing high VWF levels (Lisman et al. 2006b; Hugenholtz et al. 2013). Also, decreased levels of ADAMTS13 that are frequently present in patients with liver diseases (Uemura et al. 2008) may promote platelet function as ADAMTS13 not only processes freshly released VWF but also cleaves VWF in a growing platelet thrombus (Shida et al. 2008).

Not all these lines of evidence are conclusive. Elevated plasma levels of soluble P-selectin and PF4 may reflect decreased clearance of these markers rather than increased production. In addition, the net effect of thrombocytopenia, anemia, and high VWF on platelet function has been poorly studied. Nevertheless, several clinical observations are consistent with a relatively well-preserved function of primary hemostasis in liver diseases. First, patients with liver diseases are not protected from arterial thrombosis (Marchesini et al. 1999), which may be expected when platelet function would be substantially inhibited. Second, normalization of the platelet count in patients with cirrhosis by thrombopoietin appears to increase the risk of thrombosis (Afdhal et al. 2012), which may be related to a normal platelet count in the context of a substantial VWF/ADAMTS13 unbalance (Lisman and Porte 2012). Third, large invasive procedures in patients with cirrhosis, notably liver transplant surgery, are possible without preoperative correction of the platelet count and without perioperative administration of platelet concentrates (Massicotte et al. 2012). Nevertheless, one study has shown that severe thrombocytopenia (defined as a platelet count $<75{,}000/\mu l$) is a risk factor for procedural bleeding (Giannini et al. 2010). In addition, an in vitro study demonstrated that the thrombin-generating capacity of platelet-rich plasma from patients with cirrhosis only decreases compared to that of healthy controls when the platelet count falls below 60,000/μl. This study has been used by clinicians to advocate platelet transfusions prior to invasive procedures in patients with a platelet count $<60{,}000/\mu l$ (Northup and Caldwell 2013), thereby ignoring the fact that the function of platelets in cessation of bleeding is much more than providing a surface to support thrombin generation.

In aggregate, although platelet function defects in cirrhosis have been very well established using classical aggregometry and other static assays, accumulating evidence suggests that the primary hemostatic system in cirrhosis is relatively balanced (Violi et al. 2011) as the relatively mild thrombocytopenia is counteracted by a VWF/ADAMTS13 unbalance (Table 1). Furthermore, flow-based assays do not confirm the platelet function defects as detected in static assays, and in vivo evidence of platelet hyperactivation exists. Nevertheless, there is some clinical evidence that thrombocytopenia is related to an increased risk of procedure-related bleeding (Giannini et al. 2010). Importantly, coagulopathy, as defined by an increased international normalized ratio, appears not to be related to procedural bleeding risk (Giannini et al. 2010). Whether the bleeding risk in patients with thrombocytopenia and preserved coagulation is lower than in patients with

Table 1 Factors determining the balance in primary hemostasis in patients with cirrhosis

Factors impairing primary hemostasis	Factors supporting primary hemostasis
• Thrombocytopenia • Anemia • Platelet function defects • Reduced functional capacity of VWF • Increased production of nitric oxide and prostacyclin in vivo • Abnormal high-density lipoprotein • Increased plasma bile salts	• Substantially elevated levels of VWF • Decreased plasma levels of ADAMTS13 • Low-grade in vivo activation of platelets by activated endothelium or during infection/inflammation

thrombocytopenia and coagulopathy is uncertain and requires further study.

Management of Primary Hemostasis in Liver Diseases

The new insights in the balance of the primary hemostatic system may have consequences for management of bleeding. It has long been common practice to prophylactically correct a reduced platelet count prior to invasive procedures by infusion of platelet concentrates. More recently, thrombopoietin receptor agonists have been shown to successfully increase the platelet count in patients with cirrhosis (McHutchison et al. 2007; Afdhal et al. 2012). Given the potential compensation of the thrombocytopenia of liver diseases by elevated VWF and decreased ADAMTS13, the lack of evidence for a beneficial effect of prophylactic platelet transfusions in this setting (Tripodi et al. 2013; Yates and Sarode 2014), and the clear side effects of platelet concentrates, a restrictive approach toward prophylactic platelet transfusion in these patients appears justified. Both in the setting of liver transplantation in patients with cirrhosis and in patients with acute liver failure, it has been shown that administration of platelet concentrates was associated with poor outcome (de Boer et al. 2008; Pereboom et al. 2009; Stravitz et al. 2013a), and in liver transplantation this association appeared independent of severity of disease and other potential confounders. These potential effects of prophylactic platelet transfusion on outcome may relate to general transfusion reactions, volume overload, and transfusion-associated acute lung injury. Prophylactic administration of thrombopoietin receptor agonists should also be performed cautiously due to the potential thrombotic risk (Afdhal et al. 2012). Importantly, thrombopoietin receptor agonists were, in a randomized trial on preprocedural correction of platelet counts, associated with an increased risk of portal vein thrombosis but were not effective in reducing bleeding risk despite marked improvements in platelet count (Afdhal et al. 2012). Although some authors and guidelines advise to prophylactically administer platelet concentrates for high (er)-risk procedures (Northup and Caldwell 2013; Patel et al. 2012), a cautious use of this approach and a careful assessment of risks and benefits appear warranted. Our center promotes a wait-and-see approach for most invasive procedures, except those procedures during which bleeding can lead to irreversible damage (Weeder et al. 2014). In other words, only in case of active bleeding, blood product transfusion will be initiated. Nevertheless, although this approach is supported by an increasing number of centers that will start a liver transplant procedure without prophylactic correction of abnormal routine indices of hemostasis, clinical studies on the safety and efficacy of a restrictive strategy toward platelet transfusion in other settings are recommended (Lisman and Porte 2010).

1-Deamino-8-D-arginine vasopressin (DDAVP) is used in some centers to improve primary hemostasis in patients with cirrhosis. Although DDAVP has been shown to correct the skin bleeding time in patients with cirrhosis (Mannucci et al. 1986; Cattaneo et al. 1990; Burroughs et al. 1985), there is little evidence for clinical efficacy. DDAVP did not reduce blood loss during liver transplantation (Pivalizza et al. 2003) and had no effect on acute variceal bleeding (de Franchis et al. 1993). A randomized controlled study suggested that DDAVP is as effective as transfusion of blood products in preventing bleeding in patients (Stanca et al. 2010). However, a drawback of this study was the absence of a nontreated control group, which makes it impossible to determine whether DDAVP and blood products are equally effective in preventing blood loss or that neither intervention is effective. It is difficult to conceptualize how DDAVP could be hemostatically active in patients that already have substantially elevated VWF levels. Indeed, DDAVP infusion showed very little effect on laboratory indices of primary hemostasis including flow-based platelet adhesion studies (Arshad et al. 2015), although there is no consensus in literature on whether DDAVP actually increases VWF levels in patients with cirrhosis.

Part B: Platelets as Diagnostic Aid and Prognostic Indicator in Liver Diseases

The platelet count has been used to score various aspects of liver disease and has some prognostic value in various settings. Due to its wide availability and its low cost, the platelet count has been extensively studied in the context of disease staging and prognosis. Recent data in chronic or acute liver failure will be reviewed below.

Fibrosis and Cirrhosis

Staging of advanced fibrosis and cirrhosis is key in patients with chronic liver disease to identify those patients that require treatment. Although liver biopsy is the gold standard for disease staging, the procedure is invasive, expensive, not without risk, and may be biased by sampling errors and subjective interpretation. Noninvasive tests as an alternative for liver biopsy have been extensively studied. Both imaging techniques and clinical scores based on laboratory values have been explored (Sharma et al. 2014). A number of clinical scores include the platelet count. For example, the AST-platelet ratio index (APRI) has been extensively validated in chronic hepatitis and is useful in predicting the presence of cirrhosis in these patients (Xiao et al. 2015;

Chou and Wasson 2013). The World Health Organization has recently recommended the use of APRI to detect cirrhosis and advanced fibrosis in resource-limited settings (http://www.who.int/hepatitis/publications/hepatitis-b-guidelines-policy/en/). The APRI, however, has not been properly validated in patients with other etiologies of chronic liver disease and appears to have lower diagnostic value than other biochemical indices, particularly in alcoholic liver disease (Naveau et al. 2009). The FIB4 score consists of age, AST, ALT, and platelet count and has been proven useful in predicting fibrosis in hepatitis C and nonalcoholic fatty liver disease (Sterling et al. 2006; Shah et al. 2009). Again, the FIB4 score appears less useful in alcoholic liver disease.

The platelet count on its own has also merit in predicting whether a patient with liver disease is likely to have cirrhosis (Udell et al. 2012) or to assess severity of fibrosis (Giannini et al. 2006). In patients with established cirrhosis, the platelet count to spleen diameter ratio is potentially useful to identify those patients with esophageal varices (Chawla et al. 2012). The platelet count is also associated with the outcome of cirrhosis (decompensation or death) (D'Amico et al. 2006; Qamar et al. 2009), and it has been demonstrated that the degree of thrombocytopenia correlates to the extent of liver atrophy (Bleibel et al. 2013).

Acute Liver Failure

Recent analyses of data from the acute liver failure study group from the United States have demonstrated that patients with a poor outcome of their disease develop a more profound thrombocytopenia in the first week after admission (Stravitz et al. 2016). Specifically, platelet counts were lower in patients that died or underwent liver transplantation compared to those who spontaneously recovered. In addition, platelet counts in the first week of admission were lower in patients who developed the systemic inflammatory response syndrome, in patients who required vasopressors or renal replacement therapy, and in patients that developed hepatic encephalopathy. Interestingly, the PT/INR was not predictive of development of the systemic inflammatory response syndrome, requirement for vasopressors or renal replacement therapy, or development of hepatic encephalopathy, although it was associated with poor outcome. A possible mechanism explaining the association between thrombocytopenia and poor outcome involves platelet activation, likely initiated by the systemic inflammatory response syndrome. It has been demonstrated that exceptionally high levels of highly procoagulant microparticles are found in circulation in patients with acute liver failure, in particular in those with systemic inflammation (Stravitz et al. 2013b). From these data it has been proposed that inflammation-mediated platelet activation with subsequent platelet fragmentation to microparticles explains the relation between thrombocytopenia and outcome. In addition, the procoagulant microparticles have been proposed do drive progression of disease by enhancing intrahepatic activation of coagulation (see part D of this chapter).

Part C: Platelets as Stimulators of Liver Regeneration

Unlike other bodily organs such as the heart or kidneys, the liver has a unique regenerative capacity (Michalopoulos and DeFrances 1997). In most adult solid organs, injury tends to result in scarring and functional impairment rather than regeneration and repair. The liver is an exception to this rule, and full restoration of functional liver mass can occur even after substantial injury. The ultimate regenerative response of the liver occurs after a partial liver resection. Up to 70 % of liver tissue can be safely removed in patients that require a partial liver resection for removal of a liver tumor (Clavien et al. 2007). Following a resection, the liver eventually regenerates to its original size. In humans, substantial regeneration has already taken place after one week, with regeneration virtually complete after three to six months (Kele et al. 2012). In rodents, liver regeneration following a partial liver resection is even faster, with complete regeneration in mice after ~5 days. The liver not only regenerates following physical removal of liver tissue, but regenerative responses occur also when liver tissue is damaged by chronic or acute liver injury or by ischemia/reperfusion injury. In the context of liver transplantation, livers also suffer from acute hepatocellular injury as a consequence of combined warm and cold ischemia and the consequent reperfusion injury. The regenerative capacity of the liver following damage from either a chronic or acute insult is remarkable. Patients with acute liver failure can spontaneously recover from their disease, showing little or no evidence of histological abnormalities within months after the disease. Livers that have suffered substantial damage in the process of transplantation can also fully recover (Brenner 2009). Animal experiments and biopsy studies in humans have demonstrated that moderate fibrosis can resolve when the trigger is removed or when successful treatment is given.

Multiple laboratories have shown that platelets are vital for liver regeneration after a partial liver resection in small animal models (Lesurtel et al. 2006; Murata et al. 2008; Myronovych et al. 2008; Shimabukuro et al. 2009). Significantly, liver regeneration after partial liver resection was shown to be substantially delayed in mice that were treated with drugs that inhibit platelet function or mice that were treated with chemotherapeutic drugs or platelet-depleting

antibodies, causing almost complete thrombocytopenia. Conversely, in mice which the platelet count was increased by treatment with thrombopoietin receptor agonists, liver regeneration was accelerated (Shimabukuro et al. 2009; Lesurtel et al. 2006). Platelets appear also relevant for liver regeneration in humans as a low platelet count following a liver resection or liver transplantation is associated with postoperative liver dysfunction and mortality, which may be related to a failed regeneration (Alkozai et al. 2010). In addition to the role of platelets in regeneration following a liver resection, it has been established that platelets delay fibrosis in models of chronic intoxication (Watanabe et al. 2009) and that platelets facilitate repair after ischemia/reperfusion injury (Nocito et al. 2007). However, not all studies agree, and there is in fact also evidence that platelets are harmful in various models of liver disease, which will be discussed in part D of this chapter.

The mechanisms underlying platelet-mediated liver regeneration are poorly understood, and some of the theories discussed in literature are based on circumstantial evidence. Plausible theories and the experimental deficits will be discussed below and are depicted in Fig. 2.

Delivery of Growth Factors

It has long been assumed that the role of platelets in stimulating liver regeneration hinges strongly on growth factors stored within platelet granules. Following a partial liver resection in small animal models, a rapid influx of platelets in the liver sinusoids has been observed (Murata et al. 2007). Platelets also cross the endothelial lining within the liver and end up in the space of Disse or even within hepatocytes (Kirschbaum et al. 2015). This rapid platelet accumulation in the liver has been postulated to result in excretion of growth factors which enhance the regenerative response. Such growth factors include hepatocyte growth factor, vascular endothelial growth factor, insulin-like growth factor, and serotonin. Indeed, these mitogens have been shown to enhance proliferation of cultured hepatocytes in vitro (Matsuo et al. 2008; Balasubramanian and Paulose 1998). Compelling evidence for a role of serotonin in mediating platelet-mediated liver regeneration was provided by experiments using mice deficient in circulating serotonin (Lesurtel et al. 2006). However, the defective regeneration in these mice may also be explained by a defect in secondary

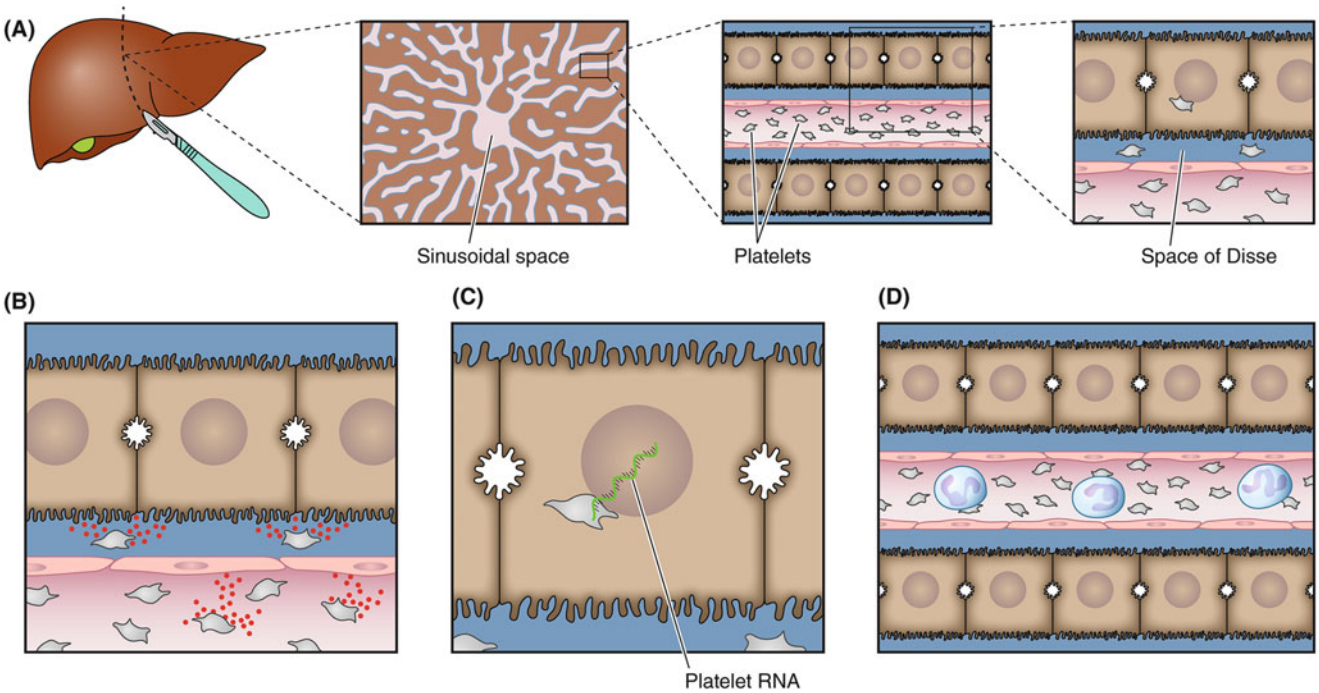

Fig. 2 Potential mechanisms underlying platelet-mediated liver regeneration. (**a**) Platelets accumulate in the sinusoidal space of injured livers or livers that have been surgically reduced in size. From the sinusoidal space, some platelets migrate into the space of Disse, and some platelets are taken up by hepatocytes. These platelets have the potential to activate multiple pathways that may all contribute to platelet-mediated stimulation of liver regeneration. Which of these pathways occur in vivo has not been definitively established. (**b**) Platelets may release contents from their granules that either directly stimulate hepatocyte proliferation (serotonin, IGF, HGF) or stimulate endothelial cells to release HGF (VEGF). Alternatively, the direct interaction of platelets with endothelial cells promotes release of interleukin 6 and VEGF that promote liver regeneration. (**c**) Platelets may transfer their RNA to hepatocytes, which promotes hepatocyte proliferation either by translation of mRNA or by the action of regulatory RNAs. (**d**) Platelets attract inflammatory cells, which are known to directly stimulate liver regeneration. Professional illustration by Patrick Lane, ScEYEnce Studios. Reprinted with permission from Lisman and Porte (2016)

platelet activation, as serotonin is not only a liver-directed mitogen but also a relevant platelet activator. Indeed, inhibition of the $P2Y_{12}$ receptor, which also results in defective secondary platelet activation, had similar effects as circulating serotonin deficiency. A human study provided evidence for serotonin consumption following a partial liver resection and showed that a low preoperative platelet serotonin content was associated with poor outcome (Starlinger et al. 2014). In this study, however, it was not examined whether the serotonin consumption following resection was related to this specific procedure or a consequence of a major abdominal surgical procedure in general. Indeed, a subsequent study in which serotonin content was compared between patients undergoing partial liver and pancreas resection provided no evidence for liver resection-specific consumption of serotonin (Alkozai et al. 2015).

Delivery of Platelet-Derived RNA

Since platelets are taken up by hepatocytes following a partial liver resection, it may be that platelets do not only deliver growth factors to the hepatocyte plasma membrane but also release factors involved in proliferation within the hepatocyte. An in vitro study demonstrated transfer of platelet-derived RNA to hepatocytes after platelet internalization by the hepatocyte with subsequent translation of platelet RNA to protein by the hepatocyte (Kirschbaum et al. 2015). This "functional" transfer of RNA contributed substantially to platelet-mediated hepatocyte proliferation. However, whether platelet RNA transfer contributes to liver regeneration in vivo needs to be established. Both platelet mRNA and regulatory RNAs may play a role in platelet-mediated liver regeneration, and the exact RNA species involved remain to be studied.

Platelets as Initiators of the Inflammatory Response

Although it is widely assumed that platelets stimulate liver regeneration by delivery of mitogenic cargo to the liver, there is another potential explanation for the role of platelets in liver regeneration. It has been well established that liver regeneration is associated with a localized or generalized inflammatory response. Liver regeneration is impaired in mice lacking inflammatory cells or production of proinflammatory cytokines such as TNF-α (Selzner et al. 2003; Yamada et al. 1997). Since platelets are well known to attract inflammatory cells (Stokes and Granger 2012), it is not unlikely that the role of platelet in liver regeneration is not mediated by platelets directly but indirectly by facilitation of the inflammatory response. Indeed, recent work has demonstrated that platelets are key in facilitating influx of neutrophils and repair following a sterile inflammation induced by thermal injury in a mouse model (Slaba et al. 2015).

Part D: Platelets as Modulators of Liver Disease Progression

Platelets as Drivers of Disease Progression

It has been well established that intrahepatic activation of coagulation occurs in animal models of acute and chronic liver disease (Anstee et al. 2008; Ganey et al. 2007). Activation of hemostasis might occur by apoptotic cells or may be initiated by platelet interactions with collagen, the deposition of which is the hallmark of fibrogenesis. Alternatively, decryption of hepatic tissue factor may initiate intrahepatic thrombus formation (Sullivan et al. 2013). Regardless of the initiating trigger, the hypercoagulable features of patients with cirrhosis likely stimulate the progression of the formation of such intrahepatic clots. There is accumulating evidence that intrahepatic thrombus formation is not just a consequence of the disease but rather an active player in disease progression. Initially, it was proposed that intrahepatic thrombi drive disease progression via a mechanism involving local ischemia (a phenomenon referred to as "parenchymal extinction") (Wanless et al. 1995). Later, it was proposed that cellular activation by coagulation proteases such as factor Xa (FXa) and thrombin is responsible for accelerating disease progression (Anstee et al. 2009). A randomized clinical trial has shown that anticoagulant treatment of patients with moderate to severe cirrhosis delays decompensation and death, indicating that prevention of intrahepatic activation of hemostasis may have clinical benefit (Villa et al. 2012). This single clinical study is in dire need of replication. Inhibition of platelet function or induction of thrombocytopenia has been demonstrated to delay disease progression in models of cholestatic (Sullivan et al. 2010) and non-cholestatic fibrosis (Assy et al. 2007), nonalcoholic fatty liver disease (Fujita et al. 2008), and acute liver failure (Miyakawa et al. 2015). In line with these results, a retrospective clinical study showed that the use of aspirin was associated with a decreased fibrosis progression in patients that underwent liver transplantation for hepatitis C (in whom fibrosis frequently recurs following transplantation) (Poujol-Robert et al. 2014).

Platelets have also been shown to facilitate $CD8^+$ T cell-induced liver damage in mouse models of hepatitis (Iannacone et al. 2005). Importantly, antiplatelet therapy was shown to decrease the severity of fibrosis, the development of hepatocellular carcinoma, and death in a mouse model (Sitia et al. 2012). It has been proposed that a clinical trial on the effect of aspirin on hepatocellular carcinoma

prevention may be realistic in high-risk patients (Carrat 2014). In those patients with advanced cirrhosis, however, there may be a potential bleeding risk due to the alterations in primary hemostasis (see part A of this chapter).

Platelets as Attenuators of Disease Progression

Confusingly, multiple studies have convincingly shown that platelets exert protective effects in animal models of liver disease. Part of these protective effects may be mediated by the capacity of platelets to stimulate liver regeneration, although this has been incompletely studied. Platelets have been shown to protect against cholestatic (Joshi et al. 2015; Jang et al. 2012; Kodama et al. 2010) and non-cholestatic liver disease (Watanabe et al. 2009) and also protect against other liver insults such as ischemia/reperfusion injury (Nocito et al. 2007). It has not yet been studied whether the different outcomes of experimental studies on platelets and liver disease progression depend on the model (e.g., hepatocyte vs cholangiocyte damage) or on the timing or mode of intervention (i.e., thrombocytopenia vs platelet inhibitors). In addition, the type, dose, and duration of pharmacological interventions may affect the ultimate outcome of the experiment. The potential efficacy of antiplatelet drugs in the prevention of progression of disease in humans may likewise be dependent on the severity of disease at onset of therapy, the underlying disease, and the type and dosing regimen of the drug, and these caveats should be considered in future clinical studies (Lisman 2015).

Take Home Messages

A Rational Approach to Management of Primary Hemostasis in Cirrhosis

- Patients with cirrhosis and thrombocytopenia can undergo major surgery (e.g., liver transplantation) without the requirement for any blood transfusion (Massicotte et al. 2015) as they are in hemostatic balance (Lisman and Porte 2010).
- Therefore, prophylactic transfusion of platelet concentrates is generally not indicated.
- Exceptions are severe thrombocytopenia and procedures during which bleeding can lead to irreversible damage.
- Thrombopoietin receptor agonists increase platelet count, do not reduce procedural bleeding, but increase risk of thrombosis.
- Therefore, thrombopoietin receptor agonists are likely not indicated in hemostatic management of patients with cirrhosis.
- DDAVP has little effect on laboratory indices of primary hemostasis in patients with cirrhosis and is likely not indicated.

Knowns and Unknowns on Platelet-Mediated Liver Regeneration

- Platelets stimulate liver regeneration after partial hepatectomy in rodent models.
- There is accumulating evidence of an association between a low postoperative platelet count and liver function recovery following partial hepatectomy in humans.
- Although release of platelet-derived growth factors is widely assumed to mediate platelet-dependent liver regeneration, no direct evidence in support of this mechanism exists.
- It also remains to be established whether platelet exert proliferative effects directly or indirectly via facilitating influx of inflammatory cells in the regenerating liver.

Platelets in Progression of Liver Diseases, Friends or Foes?

- Platelets contribute to progression of chronic and acute liver disease in multiple experimental animal models.
- Platelets have also been reported to attenuate disease progression in experimental animal models.
- Although antiplatelet therapy would be an attractive clinical strategy to reduce disease progression (as no alternatives are currently available), the relevance of the conflicting results in animal studies should be explored.

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Platelets and Renal Disorders

Jens Lutz and Kerstin Jurk

Abstract

Patients with chronic kidney disease (CKD) have an increased risk of thrombosis as well as bleeding episodes. Platelet activation, platelet–leukocyte conjugates and platelet-derived microparticles together with an activation of endothelial cells can all confer pro-thrombotic effects in platelets. Furthermore, platelets can contribute to the inflammatory state in patients with CKD and thus together with their thrombosis promoting effect can also contribute to the increased cardiovascular risk of these patients. The bleeding risk in patients with CKD can be augmented by increased concentrations of uraemic toxins, anaemia and altered endothelial cell function all contributing to an impaired platelet function. However, platelets can also be directly involved in the pathogenesis of renal diseases such as thrombotic microangiopathy, immune complex-mediated renal disease, anti-GBM glomerulonephritis and antibody-mediated rejection after kidney transplantation as they can promote inflammatory responses after their activation. The following chapter gives an overview about the mechanisms involved in thrombosis and bleeding promoting effects of platelets in patients with CKD as well as the mechanisms behind the direct pathogenic effects of platelets in several renal diseases.

Platelets in Chronic Kidney Disease

Patients with deteriorating chronic kidney disease (CKD) and particularly those with end-stage renal disease (ESRD) and uraemia develop disturbances of the haemostatic system. Both thrombosis and bleeding can occur in patients with CKD. During early CKD stages, the thrombotic risk is likely to be increased, while during late stages and in uraemia also, the bleeding tendency is augmented. The bleeding risk is related to disturbances of the coagulation system, but also to distinct defects of the platelets, such as impaired platelet interaction with the vessel wall, platelet aggregation and platelet-dependent coagulation. Furthermore, interactions with drugs affecting the haemostatic system during haemodialysis sessions further impair platelet function in patients with end-stage renal disease. Dialysis patients may also present with hyperreactive platelets and an increased risk of thrombosis or hyporeactive platelets and an increased risk of bleeding.

Platelets are also directly involved in the pathogenesis of glomerular diseases as they can attract other inflammatory cells to the site of tissue damage, interact with renal cells (i.e. mesangial cells) or release pro-inflammatory chemokines, cytokines and growth factors. Here, the platelet secretome and transcriptome play an important role.

Platelet Dysfunction and Risk of Thrombosis

Patients with uraemia can present with thrombotic events such as deep venous thrombosis, pulmonary embolism, vascular access thrombosis in haemodialysis patients, central

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vein thrombosis, right atrial thrombus, acute coronary syndrome, cerebrovascular event or peripheral artery occlusion (Ando et al. 2002; Lutz et al. 2014). CKD is characterized by inflammation, endothelial dysfunction and platelet activation. Increased levels of CRP, IL-1, IL-6 and TNF-α as well as of soluble adhesion molecules, such as soluble ICAM-1, VCAM-1 and P-selectin (Landray et al. 2004; Shlipak et al. 2003; Stenvinkel and Alvestrand 2002; Tripepi et al. 2005), are associated with CKD. It has been shown that endothelial cells exhibit decreased generation of vasoactive substances such as NO in patients with ESRD (Jankowski et al. 2003) which leads to less constitutive inhibition of circulating platelets via the cGMP/PKG pathway (Smolenski 2012; Walter and Gambaryan 2009). Hyperlipidaemia and hypertension represent important co-morbidities of CKD that have been shown to increase platelet reactivity via platelet scavenger receptors and CD40 ligand, respectively (Hausding et al. 2013; Zimman and Podrez 2010). However, how activated leukocytes and endothelial cells affect platelet function during the pathogenesis of CKD is poorly understood so far. Platelet hyperreactivity and activation affect primary haemostasis and coagulation, but also contribute to the inflammatory response via modulating the function of endothelial cells and leukocytes (Fig. 1). Particularly, hyperaggregability, the exposure of phosphatidylserine, shedding of platelet-derived microparticles and alteration in the platelet transcriptome and protein synthesis are implicated to contribute to the pro-thrombotic state.

Platelet–Platelet and Platelet–Leukocyte Interaction

Platelets of uraemic patients present normal concentrations of the glycoprotein (GP) IIb/IIIa ($\alpha_{IIb}\beta_3$ integrin) on their surface. These receptor complexes are responsible for the cross bridging of platelets via binding of fibrinogen or von Willebrand factor (VWF), resulting in platelet aggregation (Kaw and Malhotra 2006). Increased plasma levels of fibrinogen (Schoorl et al. 2013a) in patients with CKD may contribute to platelet hyperaggregability. In addition, plasma fibrinogen bridges activated platelets and leukocytes predominantly via the leukocyte αMβ2 integrin (Mac-1) to form hetero-cellular conjugates (Gawaz et al. 2005).

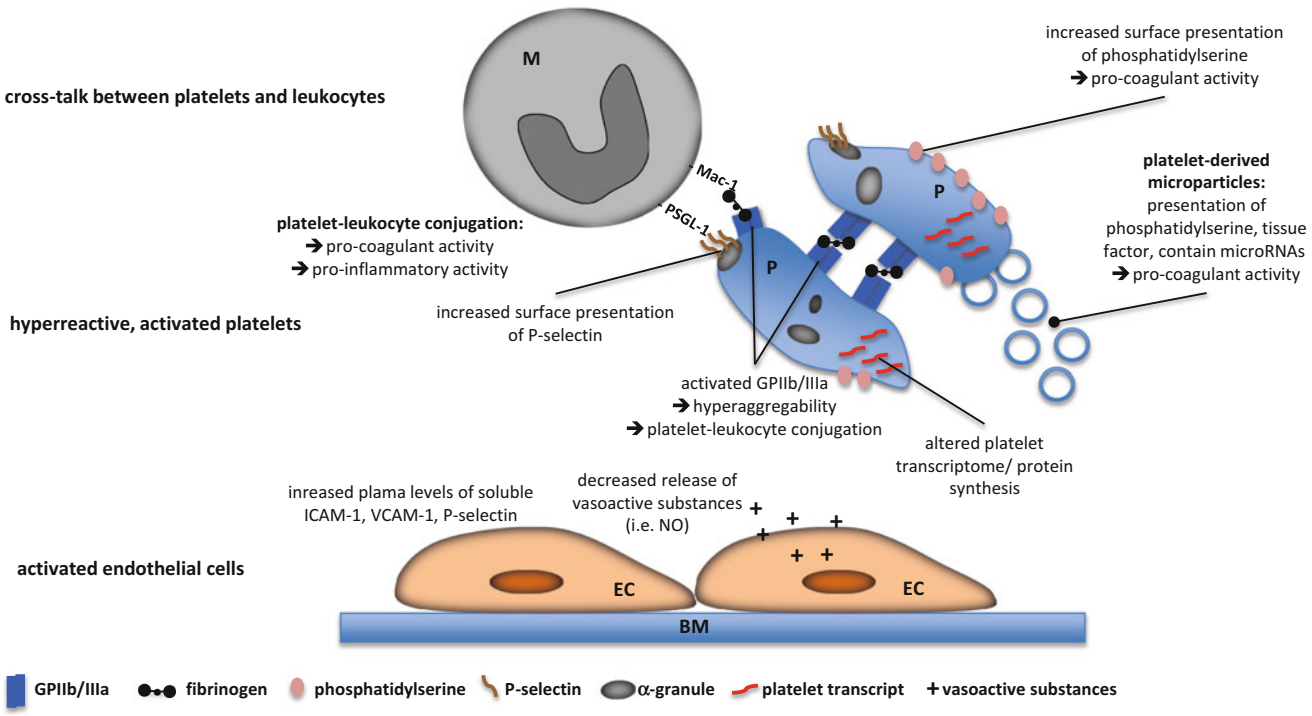

Fig. 1 Increased risk of thrombosis in patients with CKD/ESRD. Platelets from patients with CKD/ESRD especially under dialysis treatment are hyperreactive and activated. Platelet hyperaggregability, the exposure of phosphatidylserine, shedding of platelet-derived microparticles and alteration in the platelet transcriptome and protein synthesis are implicated to contribute to the pro-thrombotic state. Activated endothelial cells facilitate platelet hyperreactivity due to decreased release of NO. Activated platelets do not only interact with other platelets but also form hetero-cellular conjugates with leukocytes, such as monocytes via P-selectin/PSGL-1 and $\alpha_{IIb}\beta_3$/fibrinogen/Mac-1 adhesion axes. The crosstalk between platelets and leukocytes leads to functional interference of each other. *BM* basement membrane, *EC* endothelial cell, *M* monocyte, *P* platelet

The levels of VWF are normal in uraemic patients (Kaw and Malhotra 2006; Lee et al. 2010), but the binding to the platelets could be reduced (see below).

Platelets express increased levels of P-selectin on their surface, a marker of α-granule secretion (see also "platelet secretome") in uraemia. P-selectin-presenting platelets form conjugates with leukocytes, predominantly with monocytes and neutrophils, via the counter receptor P-selectin glycoprotein ligand-1 (PSGL-1) (Furie et al. 2001). Thereby, activated platelets modulate leukocyte function and vice versa via direct interaction supporting the pro-inflammatory and pro-thrombotic state. Soluble P-selectin shed from activated platelets or endothelial cells contributes to leukocyte recruitment to sites of vascular injury and therefore triggering the inflammatory response (Wagner 2005).

Platelet Procoagulant Activity and Exposure of Phosphatidylserine

Platelets expose increased levels of aminophospholipid phosphatidylserine (PS) on their surface that enables coordinated binding of distinct coagulation factors leading to enhanced coagulation due to amplified thrombin and subsequent fibrin formation and promoting a pro-thrombotic state in uraemia. Enhanced plasma levels of fibrinogen, VWF and FVIII, as well as decreased levels of protein C and S facilitate the generation of thrombin and fibrin generation (Galbusera et al. 2012). In addition, PS exposure on activated platelets serves as a clearance signal for macrophages (Bonomini et al. 2004, 2007) which shortens the life span of platelets in uraemia (Li et al. 2014). L-carnitine (LC) could reduce PS exposure in activated platelets in uraemia, possibly via inhibition of caspase activation (Bonomini et al. 2007). Whether uraemic patients might benefit from this effect of LC on platelet needs to be studied in the future.

Platelet Procoagulant Activity and Platelet-Derived Microparticles

Platelet-related thrombogenic factors in uraemic patients include platelet-derived microparticles (PMPs) (Goubran et al. 2015). The PMP surface is approximately 50- to 100-fold more procoagulant than the surface of activated platelets (Sinauridze et al. 2007).

PMPs are microvesicles with a diameter of 0.05–0.8 μm, a phospholipid bilayer and an increased PS exposure (Owens and Mackman 2011). They are released from the platelet membrane after activation by strong agonists, complement or shear stress as well as apoptosis. Interestingly, different activation patterns result in different PMP profiles. Strong agonists such as collagen or thrombin result in an increase of proteins which are related to platelet activation, while proteins related to platelet degranulation and the electron transport chain were reduced (Milioli et al. 2015). So far it is not clear how CKD influences platelet activation with different stimuli.

PMPs are also the result of platelet or megakaryocyte senescence as well as cytoskeletal abnormalities (Goubran et al. 2015). They influence the haemostatic balance under physiological conditions but can develop procoagulant activities resulting in thrombosis. PMPs can promote the expression of adhesion molecules, stimulate the release of cytokines, alter vascular reactivity and induce inflammation and angiogenesis as they contain growth factors, microRNA and different bioactive factors (Varon and Shai 2015). The release of tissue factor (TF) as well as coagulation factor binding to the negatively charged surface presenting PS belongs to the well-characterized procoagulant function of PMPs. In addition, PMPs promote healing and inflammatory responses. Thus, they contribute to the crosstalk and exchange of signals between cells and organs under pathological conditions (Varon and Shai 2015).

The PMPs bind factor Va and catalyse the formation of the prothrombinase complex, thus facilitating thrombosis (Sims et al. 1988, 1989). Elevated levels of PMPs were observed in cerebrovascular disease (Geiser et al. 1998) as well as acute myocardial ischaemia (Katopodis et al. 1997). Indeed, also patients with ESRD and patients on dialysis had elevated levels of PMPs, and no significant differences were observed between haemodialysis and peritoneal dialysis (Ando et al. 2002). Possibly, treatment with erythropoietin increases the release of PMPs (Ando et al. 2002). It is not clear so far what effects this has in terms of coagulation and the systemic inflammatory state of patients with ESRD.

Platelet Secretome

Platelets contain secretory granules, predominantly α-granules, δ-granules/dense bodies and lysosomes, and the release of granule cargoes is an important mechanism of platelet function and activation. Small biomolecules, such as ADP, ATP, polyphosphates, serotonin, histamine and Ca^{2+} and Mg^{2+} cations, are released from dense bodies upon platelet activation and act as important feedback and paracrine platelet agonists. Activated platelets release acid hydrolases, e.g. β-hexosaminidase, heparanase, elastase and cathepsins, from lysosomes which promote thrombus remodelling (Golebiewska and Poole 2015). However, the major cargo of platelet granules, e.g. multifunctional proteins and peptides, are stored in platelet α-granules. They contain adhesive proteins such as fibrinogen, VWF and thrombospondin-1 (TSP-1) promoting platelet

aggregation and coagulation. However, also coagulation factors, e.g. factor V and factor XIII, and anti-coagulation factors, e.g. antithrombin and proteins C and S, are stored in the α-granules. Moreover, different growth factors and chemokines such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), insulin-like growth factor 1, transforming growth factor β, platelet factor 4 (heparin-binding chemokine), fibroblast growth factor (FGF) and matrix metalloproteinases (MMPs) are stored in the α-granules of platelets (Pintucci et al. 2002; Sheu et al. 2004). Furthermore, angiostatin and endostatin, both inhibiting angiogenesis, have been located in α-granules. In addition, TSP-1 acts as an inhibitor of endothelial cell proliferation and capillary tube formation together with tissue inhibitors of matrix metalloproteinases (Golebiewska and Poole 2015). The α-granule membrane contains P-selectin but also additional receptors (e.g. $\alpha_{IIb}\beta_3$, GPIb-IX-V, GPVI) that are also constitutively expressed on the platelet surface. These are translocated to the platelet surface upon granule exocytosis and secretion mediating the crosstalk with leukocytes and the increase of receptor abundance on the platelet surface, respectively. Thus, the platelet secretome modulates not only primary and secondary haemostasis but also inflammation, cell growth and blood vessel development. So far, it is not clear how platelet secretion is affected by chronic renal failure.

Platelet Transcriptome and Protein Synthesis

Platelets do not contain a nucleus but contain cytoplasmic mRNAs as well as microRNAs derived from the megakaryocytes. This so-called platelet transcriptome influences platelet function and is altered in uraemia that seems to be at least partially correctable by dialysis (Ple et al. 2012). Moreover, it could be also a marker of disease progression (Clancy and Freedman 2015). Indeed protein synthesis can be initiated by platelet mRNAs (Schubert and Devine 2010; Schubert et al. 2014) such as the matrix metalloproteinase inhibitor TIMP 2 (Cecchetti et al. 2011). Blood clot retraction can be promoted by activated platelet-derived Bcl-3 (Weyrich et al. 2007). Interestingly the protein synthesis is altered in platelets of patients with CKD (Marques et al. 2010; Walkowiak et al. 2007). Platelet microRNAs could exert a regulatory role on platelet mRNAs. Phosphatidylcholine transfer protein (PCTP) and WD repeat-containing protein 1 (WDR1) are regulated by microRNAs. The microRNA hsa-miR-19b regulates WDR1, which is increased in platelets of uraemic patients and seems to be involved in platelet reactivity (Ple et al. 2012). It is not clear so far to what extent the platelet transcriptome influences platelet function in terms of bleeding episodes or thrombosis in patients with uraemia.

Haemodialysis

Platelet reactivity is increased in terms of fibrinogen binding in CKD patients undergoing haemodialysis (Aggarwal et al. 2002) and is associated with an increased risk of thrombosis (Kabbani et al. 2001; Thaulow et al. 1991; Trip et al. 1990) (Fig. 2). Further, increased shear stress, the surface of dialyzer membranes together with the biofilm and high- and low-molecular-weight heparin treatment may trigger platelet activation during haemodialysis (Gritters et al. 2008; Schoorl et al. 2013b). Haemodialysis results in platelet activation, characterized by granule cargo secretion with a release of platelet factor 4 as well as α- and β-thromboglobulin from α-granules together with ADP from dense bodies during dialysis with polysulfone membrane dialyzers (Elshamaa et al. 2009; Hoenich 1998). Furthermore, haemodialysis is associated with thromboxane A2 release (Hoenich 1998), platelet–monocyte and platelet–neutrophil conjugation (Bonomini et al. 1999; Gawaz et al. 1994a) and phosphatidylserine exposure of platelets (Schoorl et al. 2013b).

During haemodialysis the level of PMPs significantly increases (Daniel et al. 2006). Platelet degranulation has been shown to be associated with increased proendothelin-1 plasma concentrations from patients with end-stage CKD, a marker of endothelial cell activation (Schoorl et al. 2013a). It has been suggested that protein-bound uraemic toxins that

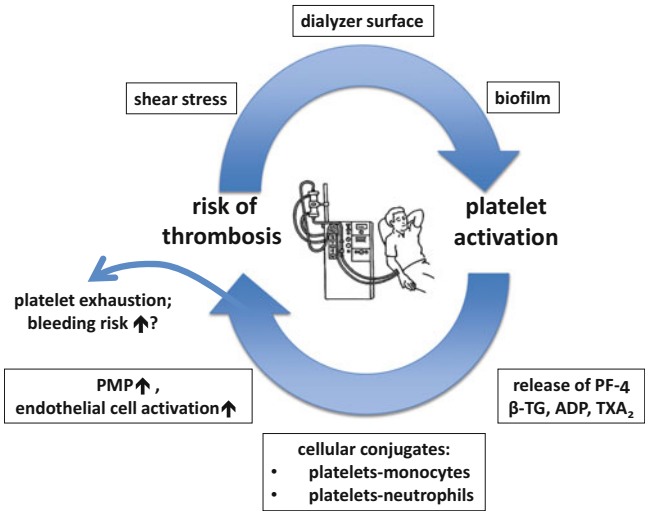

Fig. 2 Effect of haemodialysis on platelet function. Haemodialysis leads to platelet activation by an increased shear stress, the dialyzer membrane surface itself as well as the biofilm on the dialyzer membrane. Platelet activation is characterized by a release of platelet factor 4, β-thromboglobulin, ADP and thromboxane A2. Furthermore phosphatidylserine exposure on platelets increases, and platelet–monocyte and platelet–neutrophil conjugation occur. Furthermore, the level of PMPs increases. Platelet degranulation can participate in endothelial cell activation contributing to the inflammatory status in patients with ESRD

are poorly eliminated by haemodialysis cause activation of endothelial cells (Costa et al. 2008; Jourde-Chiche et al. 2009) contributing to the inflammatory status in patients with ESRD.

Platelet Dysfunction and Risk of Bleeding

Uraemic Toxins and Platelet Activation Defects in ESRD

Platelet function is substantially impaired in uraemic conditions especially in patients with ESRD (Boccardo et al. 2004; Glorieux et al. 2009) (Fig. 3). Uraemic toxins such as phenol, phenolic acid and guanidine succinic acid can mediate platelet hyporeactivity (Horowitz 1970; Horowitz et al. 1970; Rabiner and Molinas 1970). Phenolic acid can impair the primary aggregation in response to ADP, while guanidine succinic acid can inhibit the second wave of ADP-induced platelet aggregation. However, the bleeding time has not been described to correlate with the concentration of the dialyzable uraemic metabolites (Remuzzi et al. 1978a). Nevertheless, dialysis improves platelet function and reduces the bleeding risk (Benigni et al. 1993; Di Minno et al. 1985; Gawaz et al. 1994b; Sreedhara et al. 1995; Remuzzi et al. 1978b). This has been also recently demonstrated using closure time analysis for the evaluation of platelet function (Mekawy et al. 2015). Importantly, urea itself does not interfere with platelet function (Linthorst et al. 2010).

Platelets present frequently with a δ-storage pool defect characterized by a decreased content of ADP in δ-granules (Di Minno et al. 1985; Eknoyan and Brown 1981), an increased ATP/ADP ratio and a reduced content of serotonin. Platelets' ability to secrete the α-granule protein β-thromboglobulin is also diminished in patients with uraemia, all of them impairing haemostasis in these patients.

Furthermore, defective platelet release of ATP in response to thrombin together with an increased Ca^{2+} content and a reduced agonist-induced release of Ca^{2+} cations

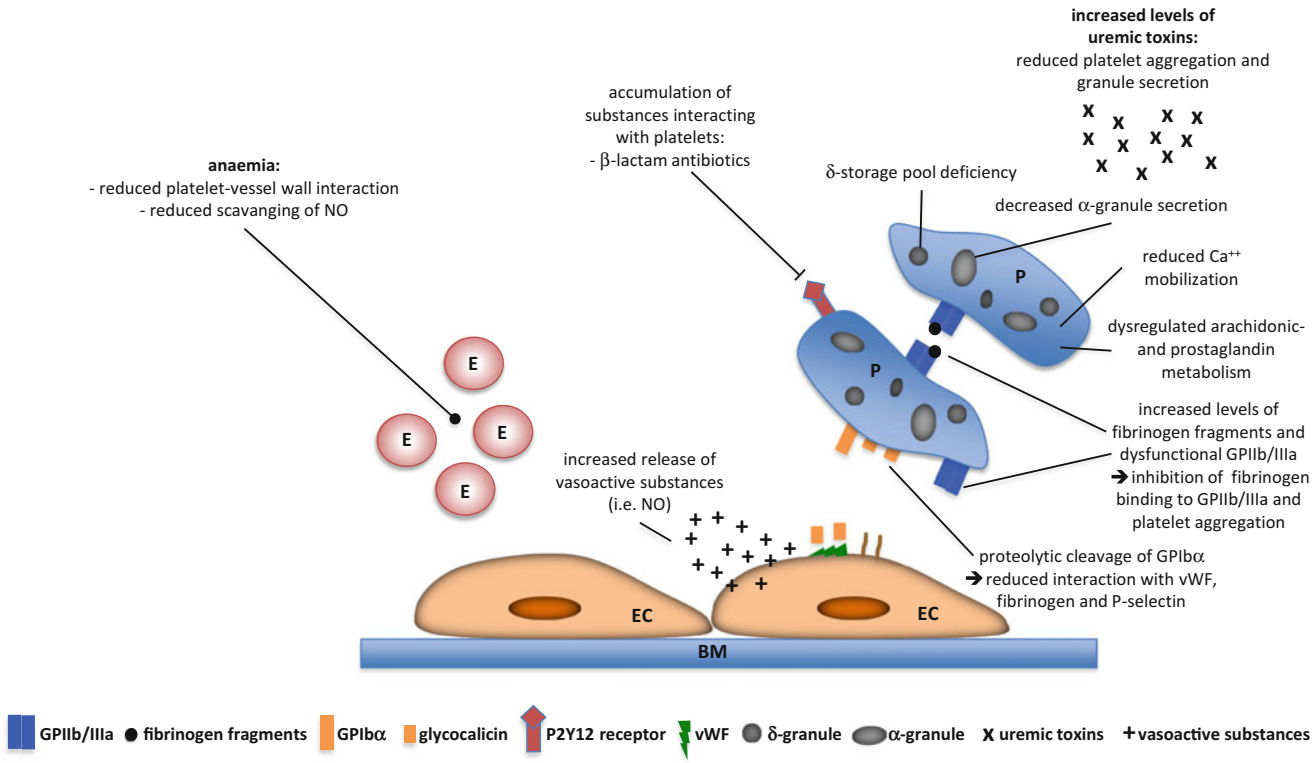

Fig. 3 Increased bleeding risk in patients with ESRD. Platelets from patients with ESRD may also present with hyporeactivity and an impaired functional response. Increased levels of uraemic toxins are predominantly responsible for decreased α-granule secretion, reduced Ca^{2+} mobilization, dysregulated arachidonic acid and prostaglandin metabolism. Platelet interaction with endothelial cells and platelet–platelet aggregation via fibrinogen binding are impaired caused by dysfunctional $\alpha_{IIb}\beta_3$ and competitive binding of fibrinogen fragments. Proteolytic cleavage of GPIbα, part of the GPIb-IX-V receptor complex, and release of the extracellular GPIbα fragment glycocalicin impair the interaction of platelets with VWF and endothelial cells. Endothelial cells especially under dialysis treatment release increased levels of NO which diminished platelet activation via the cGMP/PKG pathway. Increased NO levels are partly the result of insufficient scavenging by erythrocytes in anaemia. Further, anaemia reduces the rheological flow of platelets to the vessel wall. Platelet δ-storage pool deficiency and accumulation of β-lactam antibiotics impair platelet feedback activation through ADP-mediated signalling via the platelet ADP-receptor $P2Y_{12}$. *BM* basement membrane, *E* erythrocyte, *EC* endothelial cell, *P* platelet

from the platelets (Boccardo et al. 2004; Ware et al. 1989) could contribute to impaired platelet function in uraemia (Di Minno et al. 1985). The platelet Ca^{2+} content is also influenced by the arterial pressure, whereas lowering of arterial pressure reduces the Ca^{2+} content of platelets in patients with end-stage renal disease (Schiffl 1990). Interestingly, the release of Ca^{2+} from intracellular stores could be improved by erythropoietin (Zhou and Vaziri 2002) and in an experimental model by washing and resuspending of the platelets in non-uraemic plasma (Ware et al. 1989).

A disturbed arachidonic acid and prostaglandin metabolism associated with decreased synthesis and/or release of thromboxane A2 leads to reduced adhesion and aggregation capacity of platelets which may contribute to bleeding episodes (Di Minno et al. 1985; Smith and Dunn 1981) and could be reversed through dialysis (Bloom et al. 1986).

Clopidogrel is a potent antithrombotic drug by targeting the platelet Gi protein-coupled purinergic ADP-receptor $P2Y_{12}$ which amplifies the platelet aggregation response (Cattaneo 2015). However, it has been shown that renal dysfunction does not interfere with or aggravate the antithrombotic effect of clopidogrel (Mangiacapra et al. 2014).

Under distinct inflammatory conditions, platelets adhere through GPIbα of the GPIb-IX-V receptor complex to activated endothelial cells via binding to endothelial P-selectin (Frenette et al. 1995) or VWF (Andre et al. 2000). In addition, the major platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ mediates binding of thrombin-activated platelets to unstimulated endothelial cells via platelet-bound adhesion proteins, such as fibrinogen, and endothelial ICAM-1 (Bombeli et al. 1998). During uraemia antibody detection of platelet surface, expressed GPIbα is reduced, which could be related to its proteolytic cleavage, for example, by plasmin whose level is elevated in uraemia (Mezzano et al. 1996; Salvati and Liani 2001; Sloand et al. 1991). Further, the resulting extracellular cleavage product glycocalicin may inhibit platelet adhesion through competitive binding to VWF (Himmelfarb et al. 1998). Moreover, a functional defect in platelet–VWF interaction can be related to an increased bleeding tendency in uraemic patients (Diaz-Ricart et al. 2000; Janson et al. 1980). Platelets exhibit reduced surface expression (Moal et al. 2003) and disturbed conformational change (Gawaz et al. 1994b) of $\alpha_{IIb}\beta_3$ upon platelet activation in uraemia. A reduced binding of VWF and/or fibrinogen to $\alpha_{IIb}\beta_3$ in uraemic patients could contribute to an impaired platelet adhesion and bleeding tendency (Benigni et al. 1993). While GPIb-IX-V acts as major VWF receptor to mediate mainly platelet adhesion, $\alpha_{IIb}\beta_3$ is the major receptor for fibrinogen to mediate predominantly platelet aggregation (Jurk and Kehrel 2005). Circulating fibrinogen fragments have been demonstrated to interfere with haemostasis as they competitively bind to $\alpha_{IIb}\beta_3$ resulting in a decreased fibrinogen-mediated platelet aggregation (Wratten et al. 1999; Sreedhara et al. 1996). Therefore, decreased platelet binding of VWF and fibrinogen has central impact on the development of platelet function defects in uraemic patients. This could be related to uraemic toxins (Benigni et al. 1993; Gawaz et al. 1994b) as it can be improved by dialysis.

Platelet function as assessed by in vitro bleeding time measurement using the PFA-100 device could be restored by haemodialysis (Mekawy et al. 2015). Although shedding of GPIbα could not be influenced by dialysis, impaired agonist-induced surface expression of $\alpha_{IIb}\beta_3$ could be reversed by peritoneal dialysis as compared to haemodialysis (Salvati and Liani 2001). This points to uraemic toxins being responsible for distinct platelet functional defects. The responsible uraemic toxins include methylguanidine, guanidine succinic acid, phenols and hydroxyphenyl acetic acid (Sohal et al. 2006).

However, haemodialysis could also contribute to impaired platelet function by promoting abnormal platelet cytoskeletal assembly (Diaz-Ricart et al. 2000). This can result in deficient tyrosine phosphorylation signalling which was more prominent in HD patients as compared to CAPD patients. Interestingly CAPD even restored this phenomenon partially. In addition, recurrent platelet activation due to regular dialysis treatment causes platelet exhaustion and refractoriness leading to increased bleeding risk (Fig. 2). However, it is not clear what clinical impact these observations have in relation to the beneficial effect of HD on platelet function.

Moreover, vasoactive substances such as nitric oxide (NO), inhibiting platelet aggregation through the formation of cGMP (Noris et al. 1993), or endothelium-derived prostacyclin (PGI_2) that modulates vascular tone also inhibit platelet function in uraemia, where increased levels have been detected (Kyrle et al. 1988). Thus, elevated levels of NO and prostacyclin contribute to platelet dysfunction with an increased bleeding risk in these patients (Kyrle et al. 1988; Defreyn et al. 1980; Radomski et al. 1990; Yokokawa et al. 1995). In congruence with these findings, inhibition of NOS by L-NMMA restores also platelet activity in uraemia (Remuzzi et al. 1990; Simon et al. 1995). Moreover, renal anaemia could also contribute to higher NO levels by a reduced scavenging capacity (Noris et al. 1993), thus contributing to decreased platelet activity (Kaw and Malhotra 2006).

Anaemia

Anaemia is often present in patients with end-stage kidney disease as a result of erythropoietin deficiency. Decreased levels of erythrocytes affect indirectly platelet activity and thus can promote bleeding episodes in uraemic patients (Fernandez et al. 1985; Galbusera et al. 2009; Howard et al. 1989). In anaemia platelets flow in the middle of the bloodstream due to the lower number of erythrocytes, which

impairs the interaction between platelets and the vessel wall resulting in a prolonged bleeding time. Furthermore, the low number of erythrocytes with a reduced haemoglobin amount lead to a reduced scavenging of NO (Martin et al. 1985) thus decreasing ADP and thromboxane A2 release via an enhanced activation of guanylyl cyclase (Noris et al. 1993) with increased cGMP levels thus inhibiting platelet aggregation (Kaw and Malhotra 2006).

However, erythropoietin can also directly influence platelets (Cases et al. 1992; Farag et al. 2012). This is of particular interest as it could at least partly explain why EPO therapy aiming at normal Hb levels increases the risk of cardiovascular events and mortality in patients with chronic kidney disease (CKD). Anaemic CKD patients on ESAs demonstrated increased levels of markers of platelet activation in terms of soluble P-selectin, soluble CD40L as well as platelet microparticles (PMPs).

Drugs

Drug–platelet interactions have fundamental effects on platelet function and thus may result in bleeding tendency, which is also the case in patients with uraemia. Antibiotics such as third-generation cephalosporins and β-lactam antibiotics play a role under these circumstances (Fass et al. 1987; Shattil et al. 1980). β-Lactam antibiotics interact with platelets through an interference with $P2Y_{12}$ receptors for ADP. These effects are related to dose and duration of the therapy.

Platelet-Induced Mechanisms of Renal Disease

Platelets and Renal Damage

The vasculature, particularly the microvasculature, plays an important role in renal diseases as it can mediate tissue damage through a direct interaction with inflammatory cells but also by secreting inflammatory mediators and growth factors into the surrounding tissue. Finally, it can influence tissue homeostasis through chronic changes of the vessel wall in terms of stenosis or obstruction with resulting tissue scarring due to chronic hypoxia and malnutrition of the dependent tissue area (Couser 1994, 1998). Platelets also contribute to the disease progress as in some studies of experimental glomerulonephritis they have been shown to produce and secrete inflammatory molecules and can represent pro-inflammatory effector cells, while in other models, platelet inhibition is not effective in reducing inflammatory reactions (Barnes 1997; Barnes and Venkatachalam 1985; Johnson 1994; Johnson et al. 1991). Thrombotic microangiopathy is a classical disease where an important pathophysiologic contribution of platelets has been demonstrated. Interestingly in the concanavalin A/anti-concanavalin A mouse model of endothelial cell damage blocking the platelet $P2Y_{12}$ receptor with clopidogrel reduced platelet activation and subsequently also endothelial damage (Schwarzenberger et al. 2015). Whether this is also effective under conditions of renal failure needs further investigation. Interestingly, mice lacking the $P2Y_{12}$ receptor are also protected from capillary rarefaction as well as progression of crescentic glomerulonephritis (Hohenstein et al. 2007). Thus, platelets may be relevant mediators of renal damage.

Furthermore, platelets have also been linked to the pathogenesis of different forms of glomerulonephritis (Zoja and Remuzzi 1995). Platelets together with their degradation products have been observed within the glomeruli and other compartments of the renal tissue in patients with lupus nephritis, mesangioproliferative, membranous, or IgA nephropathy. Platelet survival is also shortened in patients with glomerular diseases that could be related to platelet consumption in some forms of glomerulonephritis (i.e. lupus nephritis) (Clark et al. 1976; Parbtani and Cameron 1980). Activated platelets may release vasoactive, chemotactic and mitogenic substances within the glomerulus that interact with resident or inflammatory cells within the kidney thus promoting glomerular injury. It has been shown that polycationic macromolecules, platelet factor 4 and β-thromboglobulin released from platelet α-granules modulate glomerular permeability and increase immune-mediated glomerular injury (Zoja and Remuzzi 1995). Platelet-derived growth factor (PDGF) (Johnson et al. 1992a) mediates renal disease progression. In an experimental glomerulonephritis model, mesangial cell proliferation and matrix expansion could be effectively suppressed by an antibody against PDGF (Johnson et al. 1992b).

Platelets can also interact with mesangial cells as has been demonstrated with activated platelets from patients with SLE (Delmas et al. 2005). The platelets induced an increased expression of CD40 on mesangial cells together with a release of soluble CD40. This was dependent from a direct contact between the platelets and the mesangial cells as well as upon platelet-associated CD40 ligand (CD40L, CD154) expression. This resulted in a proliferation of mesangial cells and an increased release of TGF-β1. Thus, platelets from patients with SLE can activate mesangial cells through CD40/CD40L signalling, leading to a proliferation of mesangial cells together with an increased production of TGF-β1. On the other hand, platelet inhibition was inferior as compared to RAS blockade in terms of reducing TGF-β expression and matrix accumulation, while it effectively reduced glomerular thrombosis in an anti-thy-1 glomerulonephritis model (Peters et al. 2004).

Inhibitors of platelet function did not clearly demonstrate positive effects in terms of reducing disease progression in clinical studies. Dipyridamole (Donadio et al. 1984;

Zimmerman et al. 1983), ticlopidin (Izumino et al. 1986; Zoja et al. 1990) and aspirin in some, partly experimental, studies in conjunction with warfarin did not show clear benefits.

Immune Complexes and Platelet Activation

IgG immune complexes can also be involved in platelet-mediated thrombotic complications that can occur during heparin-induced thrombocytopenia, systemic lupus erythematodes, rheumatoid- and collagen-induced arthritis or some forms of chronic glomerulonephritis (Zhi et al. 2015). This is related to the capability of the Fc receptor, FcγRIIa, to initiate platelet activation. On the other hand, also the major platelet adhesion receptor, integrin $\alpha_{IIb}\beta_3$, could be involved resulting in mediating adhesion and aggregation after binding to IgG immune complexes. Interestingly binding of IgG immune complexes to FcγRIIa leads to platelet adhesion and initial signal transduction; thrombus formation requires $\alpha_{IIb}\beta_3$ receptor. Furthermore, the tyrosine kinases Lyn and Syk seem to be important signal transduction molecules in IgG complex-induced platelet activation. Whether a specific inhibition improves outcomes in immune complex-mediated diseases and the impact of renal function is not known so far.

Anti-GBM Glomerulonephritis

Experiments in anti-glomerular basement membrane antibody-mediated nephritis revealed an increased expression of P-selectin on platelets (Kuligowski et al. 2006). However, their accumulation was P-selectin independent. Interestingly, platelet depletion prevented the increase in glomerular P-selectin expression as well as the leukocyte recruitment. Depletion of both neutrophils and platelets also prevented urinary protein excretion in this model of glomerular disease indicating that platelet accumulation participates in the pathogenesis of renal injury in the anti-GBM nephritis. Finally, infusion of wild-type platelets into P-selectin deficient mice restored the ability of glomeruli in these mice to support leukocyte adhesion. Thus, leukocyte adhesion in glomeruli can occur through a non-rolling interaction of leukocytes with the endothelium mediated by platelet-derived P-selectin.

Antibody-Mediated Rejection After Kidney Transplantation

Antibody-mediated rejection substantially contributes to late allograft loss (Kuo et al. 2015; Sellares et al. 2012). In an experimental model of antibody-mediated kidney graft rejection in mice administration of donor specific antibodies (DSA) resulted in C4d deposition along the capillaries and VWF release from endothelial cells coated with platelet aggregates. Platelet-derived platelet factor 4 and serotonin accumulated in the graft. Furthermore, activated platelets expressing P-selectin attached to endothelium as well as macrophages. This was associated with endothelial damage. On the other hand, platelet depletion reduced platelet-derived factors as well as the accumulation of macrophages. Thus, platelets promote inflammation in response to DSAs and could play an important role in the development of antibody-mediated allograft rejection.

Take-Home Messages

- Platelet homeostasis is substantially disturbed in patients with advanced CKD and contributes to bleeding episodes as well as to thrombosis.
- The increased risk of thrombosis is related to platelet activation, platelet–leukocyte conjugates, platelet-derived microparticles and activation of endothelial cells.
- Uraemic toxins, anaemia and altered endothelial cell function can lead to bleeding episodes.
- Platelets are involved in the pathogenesis of renal diseases such as thrombotic microangiopathy, immune complex-mediated renal disease, anti-GBM glomerulonephritis and antibody-mediated rejection after kidney transplantation.

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Platelets in Inflammatory Bowel Disease

Felix Becker and Thorsten Vowinkel

Abstract

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders of the gastrointestinal tract, which encompass two main entities: ulcerative colitis (UC) and Crohn's disease (CD). While thrombotic events have long been recognized as characteristic extraintestinal features of CD and UC, growing evidence suggests that in particular platelets play a crucial part in the integration of the interdependent processes, inflammation and thrombosis/coagulation, that are important features of these diseases. As such, platelets are suggested to be involved in modulating the intestinal inflammatory phenotype by amplifying inflammation-induced trans-endothelial leukocyte recruitment and activation. The aim of this chapter is to summarize the importance of platelet abnormalities in IBD, their relevance for extraintestinal thrombotic complications, as well as the intestinal inflammatory phenotype, and to further review recent developments in targeting platelets as a therapeutic option in treatment of patients with IBD.

Introduction

Inflammatory bowel diseases (IBD) are chronic relapsing inflammatory disorders of the gastrointestinal tract, which encompass two main entities: ulcerative colitis (UC) and Crohn's disease (CD). While thrombotic events have long been recognized as characteristic extraintestinal features of CD and UC, growing evidence suggests that in particular platelets play a crucial part in the integration of the interdependent processes, inflammation and thrombosis/coagulation, that are important features of these diseases. As such, platelets are suggested to be involved in modulating the intestinal inflammatory phenotype by amplifying inflammation-induced trans-endothelial leukocyte recruitment and activation. The aim of this chapter is to summarize the importance of platelet abnormalities in IBD, their relevance for extraintestinal thrombotic complications, as well as the intestinal inflammatory phenotype and to further review recent developments in targeting platelets as a therapeutic option in treatment of patients with IBD.

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IBD

IBD are idiopathic inflammatory conditions that represent an increasing global health issue with rising incidence in Westernized and developing countries (Molodecky et al. 2012). The principle types of IBD are UC and CD and are defined as chronic relapsing intestinal disorders, with a shared cardinal symptom of intestinal inflammation. UC is restricted to the colon and causes mucosal inflammation, whereas CD can occur along the entire gastrointestinal tract and affects all layers, termed "transmural" inflammation (Ordas et al. 2012; Baumgart and Sandborn 2012). Although the precise etiology of IBD has not yet been unraveled, it is commonly accepted that complex alterations in the mucosal immune system in genetically susceptible individuals lead to an inappropriate intestinal host response to unspecified environmental factors (Xavier and Podolsky 2007). Mounting

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Table 1 Platelet abnormalities in inflammatory bowel diseases

Quantitative
Platelet count ↑ (Morowitz et al. 1968)
PCT ↓ (Ozturk et al. 2013)
Morphological
MPV ↓ (Kapsoritakis et al. 2001)
PDW ↓ (Ozturk et al. 2013)
Granular content ↑ (Jaremo and Sandberg-Gertzen 1996)
Functional
Activation ↑ (Collins et al. 1994)
ß-TG ↑ (Vrij et al. 2000)
PF-4 ↑ (Simi et al. 1987)
sCD40L ↑ (Danese et al. 2003a, b)
s-P-selectin ↑ (Andoh et al. 2005)
PDMP ↑ (Andoh et al. 2005)
Aggregation ↑ (Andoh et al. 2006)
Homotypic (platelet–platelet) ↑ (Webberley et al. 1993)
Heterotypic (platelet–leukocyte) ↑ (Irving et al. 2004)

PCT plateletcrit, *MPV* mean platelet volume, *PDW* platelet distribution width, *ß-TG* beta-thromboglobulin, *PF-4* platelet factor 4, *s-P-selectin* soluble P-selectin, *sCD40L* soluble CD40 ligand, *PDMP* platelet-derived microparticles

evidence suggests that IBD do not just involve dysregulated immune cells but also involve complex interactions and active participation of other cellular systems, including platelet aggregates, which are frequently found within mucosal microthrombi, and contribute to the complex alterations in the mucosal immune system (Danese et al. 2004).

Platelet Abnormalities in IBD

CD and UC are both accompanied by distinct quantitative, morphological, and functional changes in platelet characteristics including increased cell number, decreased volume, enhanced activation, and elevated aggregation (Table 1). While platelet abnormalities are now a well-established feature in IBD, recent work has focused not only on unraveling a possible pathogenic role of platelets in the initiation and perpetuation of the intestinal inflammation in IBD but also on using platelets and their abnormal activity to monitor disease progression and activity in UC and CD.

Reactive Thrombocytosis in IBD

As early as 1968, Morowitz et al. first described that active IBD was associated with an increase in platelet number, a term defined as reactive thrombocytosis, and defined as a platelet count of $>450 \times 10^9$/L (Morowitz et al. 1968). Soon after that it was further demonstrated that the number of platelets correlated with recognized serological disease activity indices, including hypoalbuminemia (low albumin levels), increased erythrocyte sedimentation rate (ESR), and serum orosomucoid (an acute-phase alpha-globulin glycoprotein) concentration. These findings established platelet count as a measure of disease activity in IBD (Talstad et al. 1973; Harries et al. 1983). The unique feature of reactive thrombocytosis was also used to distinguish diarrhea, due to IBD, from infectious diarrhea, both of which can present themselves with the cardinal clinical features of diarrhea, abdominal pain, and cramping, while reactive thrombocytosis is present in IBD only (Harries et al. 1991). In addition, platelet count can even be used to discriminate between active forms of CD and UC and its inactive counterparts, as well between diseased and healthy individuals (Kapsoritakis et al. 2001). Since platelet count was positively correlated with an active state of disease in UC and CD, which predominantly represents the extent and severity of intestinal inflammation, a recent study was performed and aimed at finding an association between increased platelet numbers and colorectal neoplasia (CRN) in UC patients, as a possible consequence of exacerbated colonic inflammation. Patients with CRN had a significantly higher platelet count, a relationship that was confirmed in the corresponding univariate logistic regression analysis. Although platelet count did not reach the significance level in the subsequent multivariate logistic regression analysis, it shows the potential of platelet markers as short- and long-term serologic biomarkers in IBD (Koutroubakis et al. 2015).

Mechanisms and Consequences of Reactive Thrombocytosis

The exact mechanism behind reactive thrombocytosis in IBD is not completely understood. In human IBD, as well as experimental models of colitis, it has been shown that platelets have a normal life span, suggesting that an increased production (thrombopoiesis), rather than an accelerated activation and consumption in the inflamed microvasculature, is responsible for the elevated platelet count (Webberley et al. 1993; Senchenkova et al. 2013). In line with these findings, IBD patients with reactive thrombocytosis have increased levels of thrombopoietin (TPO), which is a crucial promoter of thrombopoiesis, as well as increased levels of interleukin 6 (IL-6), which induces hepatic TPO production. Furthermore, data obtained from a model of experimental colitis has demonstrated a connection between IL-6 and platelet activation, reactive thrombocytosis, and platelet–leukocyte aggregate (PLA) formation, with IL-6 found at least in human CD to be elevated (Yan et al. 2014; Heits et al. 1999; Senchenkova et al. 2013; Mahida et al. 1991). However, a direct correlation between serum levels of TPO and platelet count during active IBD could not be detected, which suggests that other factors might be responsible for the reactive thrombocytosis (Papa et al. 2003). While an elevated platelet count in IBD has now been well established to be

related to disease activity, thrombocytosis is not an independent risk factor for thromboembolism, as it has been found in other diseases, such as cancer (Voudoukis et al. 2014).

Morphological Platelet Changes

Although the exact mechanisms responsible for the alterations in thrombopoiesis during the active phases of disease in UC and CD patients remain unknown, one distinct feature of this process is well established in IBD, and that is the smaller mean platelet volume (MPV). Platelet number and volume are normally inversely related, and as such, studies have comprehensively shown a decreased MPV to be associated with the increased platelet count in IBD (Jaremo and Sandberg-Gertzen 1996; Chakraborty et al. 2011). Moreover, since the average size of a platelet correlates with its function and activation, efforts have been made to use MPV as an additional serologic inflammatory biomarker for the detection and activity surveillance in patients with UC and CD (Martin et al. 1983; Thompson et al. 1982; Gasparyan et al. 2011). While the feature of a reduced MPV has been uniformly described in early IBD studies, subsequent reports correlated this feature with disease activity and severity. It was reported that active CD and UC were associated with a decreased platelet volume, which was inversely correlated with traditional serologic inflammatory markers such as C-reactive protein (CRP), white blood cell count (WBC), and ESR (Kapsoritakis et al. 2001; Ozturk et al. 2013; Shen et al. 2009). An additional correlation with an endoscopic activity index was later described for UC (Yuksel et al. 2009). Similar to reactive thrombocytosis, the exact mechanism(s) responsible for the decrease in MPV in IBD remain uncertain. Early studies suggested an enhanced thrombopoiesis as a result of an increased consumption of larger platelets and the subsequent release of immature and smaller platelets (Voudoukis et al. 2014). It has also been suggested that younger platelets (with a higher MPV) are more prone to form platelet aggregates, which in turn would increase the relative appearance of older platelets with a smaller MPV (Vizioli et al. 2009; Giannotta et al. 2015). However the abovementioned studies demonstrating a normal platelet life span in IBD challenged this explanation (Webberley et al. 1993). In addition, the lower MPV in IBD patients was not correlated with levels of pro-thrombopoiesis molecule TPO but rather negatively correlated with the platelet-specific activation markers: beta-thromboglobulin (ß-TG) and platelet factor 4 (PF4) (Kapsoritakis et al. 2001). This further demonstrated the need for platelet kinetic studies in the specific setting of IBD, which take into account platelet activation, possible platelet consumption, and an altered thrombopoiesis due to the chronic inflammatory state.

Platelet Activation in IBD

In IBD, growing evidence suggests that platelets circulate in an active state, with the inflamed intestinal microcirculation being the likely origin of activation. This notion is further supported by findings that platelet activation and aggregation are increased in capillaries compared to peripheral venous blood (Tschoepe et al. 1993; Collins et al. 1997; Irving and Rampton 2007). In UC and CD, the mesenteric microvasculature is chronically inflamed, leading to continuous endothelial cell damage, indicated by increased serum levels of von Willebrand factor (vWF), endothelial protein C receptor (EPCR), and thrombomodulin (TM) (Stevens et al. 1992; Boehme et al. 1997; Meucci et al. 1999). Endothelial cell damage results in the exposure of basement membrane collagen and tissue factor (TF), which are both potent stimulants for platelet activation. The characteristic chronic intestinal inflammation in CD and UC also results in microvascular dysfunction with endothelial cell activation as well as leukocyte recruitment and transmigration. As outlined below, platelets can be directly activated by the receptor-mediated contact to stimulated endothelial cells or to leukocytes adhering to the endothelial cell layer. In addition, a variety of soluble mediators including lipid mediators (e.g., platelet-activating factor [PAF]), cytokines (e.g., interferon-γ), or chemokines (e.g., CCL22), which are released by resident or recruited immune cells and are all able to activate platelets (Stokes and Granger 2012).

Assessment of Platelet Activation

The activated state can be accessed by serum measurements of platelet-derived molecules (such as ß-TG and PF4) or by directly assessing membrane-bound activation-dependent antigens (e.g., glycoproteins $\alpha_{IIb}\beta_3$, P-selectin, CD40, and GP53) on platelets. Once activated, platelets release a broad variety of activation products and undergo distinct changes in the character and formation of their membrane-bound receptors. These effects will now be discussed in more detail.

Soluble Markers of Platelet Activation

In the context of IBD, ß-TG and PF4, both released from the platelet α-granules, have gained interest as markers of platelet activation. Since PF4 and ß-TG are also potent chemoattractants for neutrophils, monocytes, eosinophils, and fibroblasts, it was suggested that the inflammatory response in UC and CD could be modulated by the degree and endurance of platelet activation; thus efforts were made to correlate levels of PF4 and ß-TG with disease activity (Collins and Rampton 1997). While it was uniformly described that both PF4 and ß-TG were markedly raised in CD and UC, conflicting results were obtained when describing a correlation between these factors and IBD disease activity. Collins et al. and Webberley et al. found no association between platelet

activation and disease status in UC and CD, while Vrij et al. were able to correlate PF4 and ß-TG levels with biochemical and endoscopic indices of disease activity (Collins et al. 1994; Webberley et al. 1993; Vrij et al. 2000).

An additional serological marker for heightened platelet activation in IBD was introduced by Danese et al. who showed that soluble CD40 ligand (sCD40L), which is exclusively shed by activated platelets, is elevated in UC and CD. Of interest, concentrations of sCD40L in UC and CD have been described to be positively correlated with the extent of mucosal inflammation, suggesting that this platelet-derived activation marker might be a suitable serologic marker of disease activity in IBD (Danese et al. 2003a, b; Ludwiczek et al. 2003).

Recently, platelet-derived microparticles (PDMP) have been accessed as markers of platelet activation during inflammation. Evidence suggests that activated platelets in IBD shed these microparticles, and in UC and CD, PDMP formation is significantly increased. Furthermore, PDMP levels have also been shown to be both correlated with disease activity and with soluble P-selectin (a classical marker of platelet activation) (Andoh et al. 2005). Thus, these findings suggest PDMP to be promising serologic markers to measure platelet activation and disease activity in patients with IBD.

Membrane-Bound Markers of Platelet Activation

Among the platelet surface markers, growing evidence suggests GPIIb/GPIIIa, P-selectin, CD40, and GP53 to be not only selective markers for platelet activation but also functionally crucial for the inflammatory and hemostatic properties of activated platelets in IBD. We discuss each marker in more detail below.

GPIIb/GPIIIa The platelet-specific glycoproteins IIb (CD41) and IIIa (CD61), also known as integrin $\alpha_{IIb}\beta_3$ complex, undergo specific conformational changes upon platelet activation, which enables the complex to bind its respective ligands, primarily fibrinogen. GPIIb/GPIIIa is essential for platelet aggregation and endothelial adherence and especially important for the cross-linking of thrombin-activated platelets in the process of clot building, where soluble fibrinogen bridges activated platelets via GPIIb/GPIIIa binding. The importance of thrombin activation in CD has recently been defined by showing that responsiveness to thrombin was higher in patients with active CD compared to patients in remission and healthy controls (Schmid et al. 2014). An increase in GPIIb/GPIIIa-positive platelet area was found in the colonic mucosa of UC patients with active disease, which was not only significantly greater when compared to healthy controls, but also higher compared to patients with inactive UC (Kayo et al. 2006).

P-selectin P-selectin is a member of the cell adhesion molecule (CAM) family and expressed on the surface of activated platelets and endothelial cells. Its respective ligand is P-selectin glycoprotein ligand-1 (PSGL-1), which is constitutively expressed on the surface of leukocytes. Thus, P-selectin–PSGL-1 binding is necessary for platelet–leukocyte aggregates. In IBD, the expression of P-selectin is significantly increased when compared to platelets from healthy controls, although independent of disease activity (Collins et al. 1994).

CD40/CD40L The CD40/CD40L system (a member of the TNF superfamily) is one of the key pathways in IBD, since it enables the cross talk between classic immune and nonimmune cells in the initiation and perpetuation of inflammation. While CD40 is expressed on a variety of cells, the membrane glycoprotein CD40L is primarily found on the surface of activated platelets and CD4+ T cells. Functionally, platelet CD40L and platelet-derived sCD40L are crucially involved in inflammatory as well as thrombotic cascades (see below) as they are the central ligand/receptor pair for platelet–leukocyte, platelet–platelet, and platelet–endothelial cell interactions and co-stimulations (Vowinkel et al. 2007; Andre et al. 2002a, b; Danese et al. 2003a, b). Platelets from UC and CD patients have a significantly greater expression of CD40L and are in addition the exclusive source of sCD40L, which is also significantly increased in the plasma of IBD patients (Danese et al. 2003a, b).

GP53 GP53 is another surface glycoprotein, which is exclusively found on activated platelets, and whose surface appearance indicates the degranulation of lysosomes. GP53 has been described to be significantly upregulated on platelets from both CD and UC patients compared to healthy controls, while no correlation with disease activity or extent of inflammation was reported when comparing active with inactive disease (Collins et al. 1994).

Platelet Aggregation

Activated platelets can form two distinct forms of aggregates: homotypic platelet–platelet and heterotypic platelet–leukocyte aggregates (PLA). As such, platelet aggregates are involved in thrombotic as well as in inflammatory processes. Since homotypic platelet adhesion is required for the sequence of thrombus development, platelet–platelet aggregates are most likely prone to be involved in intestinal thrombus formation. On the other hand, it has been shown that leukocytes, which form a complex with activated platelets, are more intensely activated than free circulating ones (Peters et al. 1999). As a consequence of this enhanced activation complex, leukocytes display increased inflammatory properties and are more likely to adhere and subsequently migrate into inflamed tissues. While the formation of platelet–platelet aggregates is mediated by fibrinogen or vWF cross-linking via the GPIIb/GPIIIa

receptor complex on activated platelets, the formation of PLA is mostly established via binding of P-selectin (expressed on platelets and endothelial cells) and PSGL-1 (constitutively expressed on leukocytes). In addition, participation of CD40L (expressed on platelets), Mac-1 (expressed on leukocytes), as well as GPIIb/GPIIIa–fibrinogen–GPIIb/GPIIIa cross talk has also been described to be involved in the formation of PLA.

Mechanism Consequences of Platelet Aggregation in IBD

There are several lines of evidence that are consistent with platelet hyper-aggregation in IBD patients. Webberly et al. described spontaneous in vitro aggregation of platelets isolated from 30 % of IBD patients, an observation that was absent in healthy controls. In addition, platelets obtained from UC and CD patients were hypersensitive in response to aggregating agents such as adenosine diphosphate, collagen, and arachidonic acid (AA) (Webberley et al. 1993). Collins et al. described an enhanced in vitro platelet aggregation in response to AA in CD and UC patients, which was IBD specific since it was not found in patients with rheumatoid arthritis (Collins et al. 1994). It was also further shown that the enhanced hyper-aggregation of platelets in IBD continued even during inactive states of disease, suggesting a possible involvement of activated platelets in relapsing flares of IBD patients (Andoh et al. 2006). When platelet aggregates were studied in vivo, patients with active IBD exhibited more circulating aggregates than healthy controls or rheumatoid arthritis patients (Collins et al. 1994). These results suggest that the hyper-aggregation of platelets in IBD cannot be seen as a general common consequence of chronic inflammation but rather as a specific characteristic feature of CD and UC. This phenomenon was further defined by showing that IBD patients had more circulating aggregates in the mesenteric venous circulation than in the supplying arterial system, as well as intracapillary platelet aggregates in rectal biopsies, suggesting the microcirculation within the inflamed intestine as the origin of platelet activation and aggregation (Collins et al. 1997; Dhillon et al. 1992). Compared to healthy individuals as well as inflammatory controls (rheumatoid arthritis), IBD patients exhibit an increased appearance of PLA. Using peripheral whole blood samples, Irving et al. showed that formation of circulating PLA was higher in IBD patients than it was in healthy and inflammatory controls (Irving et al. 2004). Furthermore, they identified that the mesenteric vasculature was the origin of PLA formation in patients with UC (Irving and Rampton 2007). One common finding in these studies was that the formation of PLA was not correlated with disease activity, which was confirmed when specifically analyzing platelet–monocyte and platelet–neutrophil aggregates in UC patients. This dissociation between disease activity and heterotypic platelet aggregation suggests that PLA formation might be pathogenic in IBD rather than just caused by chronic inflammation.

Although platelets might represent the crucial link between the characteristic interaction between thrombosis/coagulation and inflammation in IBD, it is worth mentioning that other hemostatic parameters involved in the coagulation cascade are also altered. While some of the changes in platelet activation and aggregation are found to be IBD specific, chronic inflammation in general activates coagulants, reduces anticoagulants, and inhibits fibrinolysis, all of which most likely participate in the pro-thrombotic state associated with IBD (Senchenkova et al. 2015; Giannotta et al. 2015).

- CD and UC are accompanied by distinct quantitative morphological and functional changes in platelet characteristics, including increased cell number and decreased volume.
- Platelets in IBD are characterized by an enhanced activation and elevated aggregation.

Platelets in Extraintestinal Disease

While UC and CD primarily affect the gastrointestinal tract, a variety of extraintestinal manifestations have been described in IBD patients. Besides solid organ manifestations such as dermatologic, musculoskeletal, or ocular disease, the vascular and hemostatic systems are commonly affected, resulting in thromboembolic disease, in which platelets have shown to play a crucial role. While a variety of diseases (such as cancer) are linked to an increased risk for the development of thrombosis, neither other chronic inflammatory disorders, which are similar to IBD (e.g., rheumatoid arthritis), nor other chronic bowel diseases (e.g., celiac disease) have been shown to enhance the risk for systemic thromboembolic events, suggesting this feature to be unique for IBD (Miehsler et al. 2004).

Thromboembolic Events

Although the precise etiology of IBD-related thrombotic events is multifactorial and remains poorly understood, growing evidence suggested that the procoagulant tendency in CD and UC is most likely a result of complex abnormalities in the coagulation and fibrinolysis system, in particular alterations in the character and function of platelets (Yoshida and Granger 2009). In addition, platelet activation and aggregation are unique to IBD while not present in other inflammatory diseases; thus platelets have been implicated as cellular mediators of the distant organ thrombosis response to the intestinal inflammation in IBD (Collins et al. 1994). As described above, in patients with

UC and CD, platelets are primed to form homotypic platelet–platelet and heterotypic platelet–leukocyte aggregates in the microvasculature of the inflamed intestine and circulate in an enhanced state of activation. Therefore, formation of activated hyperaggregable platelets have been suggested to be a key event for the enhanced thrombogenic potential observed in CD and UC patients, as they are crucial for the onset and stabilization in the sequence of thrombus formation (Fig. 1) (Senchenkova et al. 2015).

The association between thromboembolic (TE) events and chronic intestinal inflammation was first described in 1936 by Bargen et al. who reported venous and arterial thrombosis as a serious complication in patients with UC (Bargen and Barker 1936). Since then, a mounting body of evidence has established IBD patients to have a greater risk for TE events, with reported incidences in clinical studies ranging from 1.2 to 7.5 % (Talbot et al. 1986; Webberley et al. 1993). These numbers even rise up to 41 % when postmortem autopsy studies are analyzed (Sloan et al. 1950). In recent years, cohort studies from Austria and Canada have also shown that the overall incidence of TE events is around 6.5 % in both UC and CD patients, with a threefold increase in the risk of systemic TE events compared to the general population and matched inflammatory diseases (Miehsler et al. 2004; Bernstein et al. 2001). In IBD, both the arterial and venous segments of the vascular tree are affected by thromboembolic complications, although thrombus formation in the venous system is more frequent. Most TE events become manifested as deep venous thrombosis (DVT) or pulmonary embolism (PE); however, less frequently affected sites include mesenteric, portal, or retinal veins; the cerebrovascular system; as well as arteries of the lower and upper extremities, renal or coronary arteries. TE events are among the leading causes for morbidity and mortality amid UC and CD, and in the case of TE events associated with IBD patients, their mortality is twofold higher than in non-IBD patients (Nguyen and Sam 2008).

Mechanisms of Thromboembolic Events

While the enhanced thrombus formation in IBD patients within the inflamed intestinal microvasculature can be in part explained by direct inflammation-induced disturbances to the vascular endothelial cell layer, such as a loss of EPCR and TM, these factors cannot account for thrombotic events observed in distant vascular beds (Scaldaferri et al. 2007). Thus, mechanistically, it has been suggested that platelets are primed for activation and aggregation in the inflamed intestinal

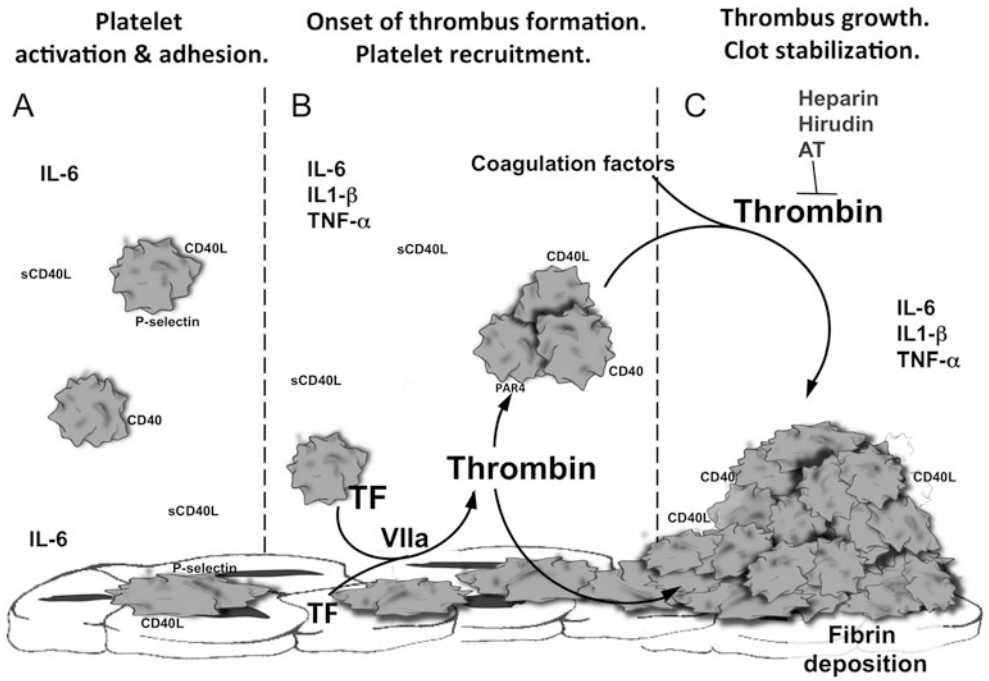

Fig. 1 Sequential changes in platelet and endothelial cell function that lead to thrombus formation in the vasculature during colitis. (Panel A) Different mediators released from the inflamed bowel elicit the activation of platelets, which promotes their adhesion to vascular endothelium via a CD40L and P-selectin-dependent mechanism. (Panel B) The accumulation of inflammatory cytokines (IL-6, IL-1β, TNF-α) results in the expression of TF and the subsequent generation of factor VIIa and thrombin, which promotes the recruitment of additional adherent platelets and the onset of thrombus formation. (Panel C) The rapid accumulation of platelets and deposition of fibrin result in thrombus growth and stabilization on the vessel wall. The transition from B to C is impeded by the antithrombin agents, heparin, hirudin, and antithrombin. *IL* interleukin, *TF* tissue factor, *TNF-α* tumor necrosis factor-α [Reproduced with kind permission of *Seminars in Thrombosis and Hemostasis* and the author (Senchenkova et al. 2015)]

microcirculation and then form thrombi in remote vascular beds. In line with this idea are observations from recent clinical studies with IBD patients, which suggest that TE events are more frequent during active disease state and that their incidence correlates with the extent of disease, with a shift toward TE events in CD during colonic involvement and in UC with pancolotic involvement (Solem et al. 2004; Miehsler et al. 2004; Spina et al. 2005; Oldenburg et al. 2000, 2005). It is however notable that approximately a third of all TE events in IBD occur while the disease is quiescent (Talbot et al. 1986).

A similar mechanism for an intestinal release and distant pro-thrombotic effects has been shown in experimental models of colitis for common soluble inflammatory mediators such as IL-1ß, TNF-α, and IL-6. All three cytokines were elevated during murine colitis and were able to mimic IBD-related extraintestinal thrombosis when administered to WT mice (Yoshida et al. 2010, 2011; Yan et al. 2014; Senchenkova et al. 2013). These observations indicate that intestinal inflammation and extraintestinal thrombosis are intrinsically linked. Following on from these findings, recent studies have provided further evidence for the role of platelets and specifically the CD40/CD40L signaling pathway as the key biological mediators involved in this cascade. Soluble or cell membrane-bound CD40L is expressed and shed by platelets activated in the inflamed mucosa, increased in UC and CD patients, and involved in the linkage between inflammation and thrombosis/coagulation in IBD (Danese et al. 2003a, b; Koutroubakis et al. 2004; Vowinkel et al. 2007). Elevated CD40L is a risk factor for vascular events, can bind to GPIIb/GPIIIa, and elicits pro-thrombotic capacity by stabilizing arterial thrombi and stimulating vascular endothelial cells to induce the upregulation of TF, which is an important initiator of IBD-related thrombus development (Anthoni et al. 2007; Andre et al. 2002a, b; Slupsky et al. 1998; Prasad et al. 2003). In addition, Gavins et al. suggested that activated and adherent platelets within the inflamed microvasculature of experimental colitis are the source of heightened levels of sCD40L, which then subsequently contribute to the enhanced incidence of thrombotic events in distant organs, as their work revealed that CD40L and sCD40L are crucial for intestinal inflammation-enhanced thrombosis in remote microvascular beds (Gavins et al. 2011).

- In patients with UC and CD, platelets are primed to form homotypic platelet–platelet and heterotypic platelet–leukocyte aggregates in the microvasculature of the inflamed intestine and circulate in an enhanced state of activation.
- As a consequence, IBD patients are prone to develop thromboembolic events

Platelets in Intestinal Disease

Initially platelets were recognized to be engaged solely in coagulation and hemostasis. However, a growing body of evidence has demonstrated their direct involvement in the mucosal immune response and inflammatory cascade in IBD. The suggested function of platelets within the complex mucosal immune system has been derived from their capacity to liberate a broad range of pro-inflammatory molecules, which can activate leukocytes and vascular endothelial cells. On the other hand however, platelet activation can also be elicited by substances released from both inflammatory cells and endothelial cells themselves (Fig. 2).

Platelet–Platelet Interactions

Convincing results have recently expanded the role for platelet activation and aggregation in IBD and even identified the diseased intestine as origin of such activity. Although the precise molecular mechanism(s) remain poorly understood, several pathways have been suggested to be involved in the activation of platelets and the cross talk to immune and other nonimmune cells within the inflamed microvasculature in IBD. Platelets and their activation products (e.g., histamine, thromboxane A_2 [TXA_2], PF-4, ß-TG) stimulate vascular endothelial cells and leukocytes in the inflamed microvasculature and therefore are able to locally intensify acute and chronic inflammatory conditions. In addition, intestinal micro-infarction, as the result of enhanced platelet aggregation, has been suggested to be involved, at least in part, in the pathogenic process associated with CD.

The initial recognition for an involvement of activated platelets in the mucosal pathology in IBD has arisen from histological studies, which revealed the presence of mucosal thrombi in rectal biopsies of CD and UC patients. Although not directly related to disease activity, this specific feature of capillary thrombi was not found in control biopsies (Dhillon et al. 1992). These intravascular thrombi within mucosal capillaries are formed of activated platelets, which are connected via GPIIb/GPIIIa–fibrinogen cross-linking and thought to be responsible for the observed multifocal mesenteric micro-infarction in CD (Sankey et al. 1993). This notion was further supported when it was discovered that patients with CD exhibit an increase in the procoagulant molecule TF, which correlates with the extent of thrombosis in the mucosal vascular bed (More et al. 1993). The increase of TF levels was due to an upregulation of production by monocytes, macrophages, and vascular endothelial cells, an effect which was later found to be dependent on CD40L binding (Slupsky et al. 1998). A direct functional consequence of this intestinal micro-infarction is the impaired mucosal perfusion, which has

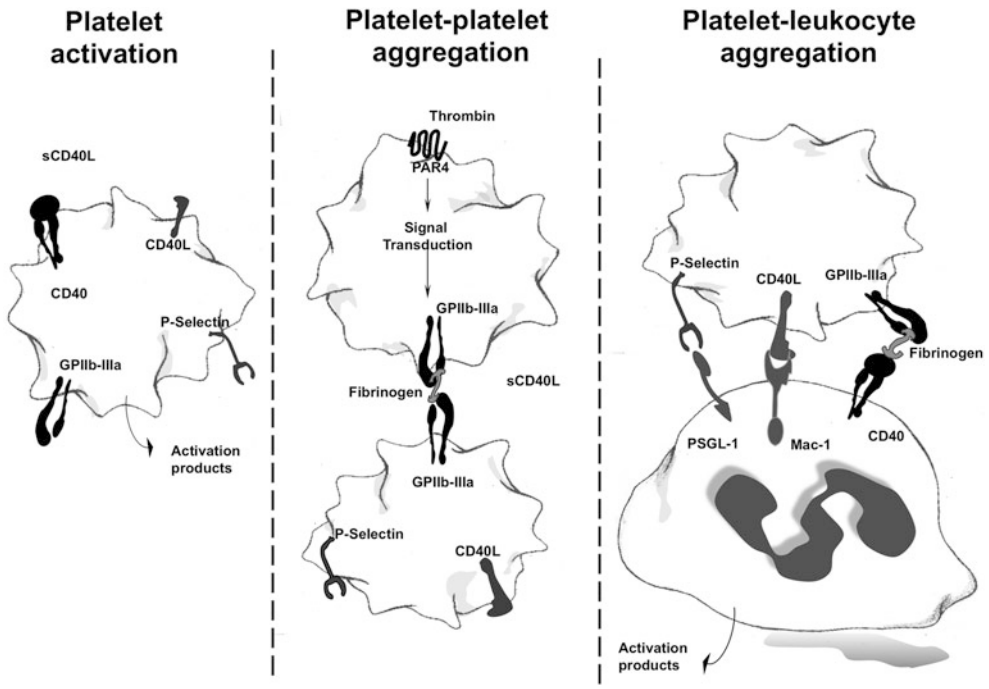

Fig. 2 The diverse platelet responses that accompany human and experimental colitis. Mediators released from the inflamed bowel predispose platelets to activation, platelet–platelet aggregation, and platelet–leukocyte aggregation. Platelet–platelet aggregation results from the bridging of GPIIb/GPIIIa on thrombin-activated platelets via fibrinogen. The heterotypic aggregation of platelets with leukocytes is mediated by three types of platelet–leukocyte adhesive interactions, that is, P-selectin–PSGL-1, CD40–Mac-1, and GPIIb/GPIIIa–fibrinogen–GPIIb/GPIIIa [Reproduced with kind permission of *Seminars in Thrombosis and Hemostasis* and the author (Senchenkova et al. 2015)]

been suggested to be involved in the pathogenesis of the progressive destructive inflammation associated with IBD (Hatoum et al. 2003; Wakefield et al. 1989).

Platelet–Endothelial Cell Interactions

Endothelial cells are the origin of platelet activation at the tissue level but are also the target of a variety of platelet-released activation products. Thus, on the one hand, the endothelium activates platelets, but on the other hand, endothelial cells can be stimulated by activated platelets. As described above, early in vivo evidence suggested that platelet activation in IBD takes place in the vast intestinal microvascular bed of the inflamed mesenteric endothelium (Collins et al. 1997). This concept was further supported recently in an in vitro study in which human intestinal microvascular endothelial cells (HIMEC) were co-cultured with the inflammatory cytokine IL-1ß to mimic the inflamed mucosa conditions in IBD. Once exposed to that inflammatory milieu, activated HIMEC triggered platelet activation, as observed by an increased CD40L and P-selectin surface expression, as well as a significantly increased release of sCD40L. In addition, in the clinical setting, it was shown that the sCD40L levels in UC and CD were proportional to the anatomical extent of mucosal inflammation, which reflects the size of the activated endothelial cell surface area encountered by platelets (Danese et al. 2003a, b). While these results demonstrated that endothelial cells are able to activate platelets to express and release CD40L, the same pathway has been shown to be involved in the stimulation of endothelial cells by activated platelets. Clinical findings have demonstrated that CD40L+ platelets adhere to the intestinal microvascular endothelium in IBD patients, forming intravascular platelet aggregates. Downstream effects of this cross talk have been shown in vitro, where activated CD40L+ platelets from IBD patients induced an upregulation of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), as well as production of IL-8 by HIMEC in a CD40-dependent fashion (Danese et al. 2003a, b). The upregulation of ICAM-1, VCAM-1, and IL-8 is a key step in the pathophysiology of IBD, since IL-8 is one of the main neutrophil chemoattractants, and CAMs are necessary for the hallmark events of leukocyte adhesion and trans-endothelial transmigration in the inflamed mucosa. Additionally, on binding to HIMEC, platelets are also able to release large amounts of RANTES, which in turn binds to glycosaminoglycans and is then presented on the surface of HIMEC, increasing their adhesiveness for T cells. Furthermore, this release of RANTES is further exacerbated in activated platelets from UC and CD patients (Danese et al. 2003a, b). Data from experimental colitis models also suggests that activation-specific platelet-derived factors such as histamine, PAF, and cationic proteins directly

contribute to endothelial cell dysfunction (e.g., increased vascular permeability) (Mori et al. 2005a, b).

Platelet–Leukocyte Interactions

Besides the abovementioned capacity to prone endothelial cells for an enhanced leukocyte recruitment, adhesion, and chemotaxis, activated platelets in IBD can also directly interact with immune cells to induce a pro-inflammatory phenotype and stimulate their adhesiveness. Such a pro-inflammatory stimulation was shown in UC patients, where activated platelets enhanced the production of reactive oxygen species (ROS) in neutrophils, which at least in part may be partially responsible for the well-established neutrophil-mediated mucosal damage in UC (Suzuki et al. 2001). On the other hand, when leukocytes are recruited to sites of injury in the intestinal microcirculation, they in turn can activate platelets by releasing PAF followed by the release of TXA_2 and subsequent platelet activation and aggregation (Eliakim et al. 1988; Webberley et al. 1993). The co-dependence of leukocyte recruitment and platelet activation in IBD was demonstrated by Vowinkel et al. in an experimental model of colitis. In this study, they showed that in the inflamed colonic microvasculature, the majority of leukocytes that were adherent to endothelial cells were attached to platelets. The importance of the P-selectin–PSGL-1-mediated binding and co-stimulation was further shown when platelet depletion diminished the recruitment of leukocytes in this in vivo model (Vowinkel et al. 2007).

- In IBD, platelets are directly involved in the mucosal immune response and inflammatory cascade.
- As such, it has been suggested that platelets play a role in modulating the intestinal inflammatory phenotype by amplifying inflammation-induced transendothelial leukocyte recruitment and activation.

Platelet-Directed Therapies in IBD

It has become evident that platelets are a crucial link between the two interdependent cascades, thrombosis/coagulation and inflammation, in IBD. Growing evidence suggests that the cross talk between platelets and other nonimmune cells, as well as classic immune cells, is in part responsible for the development of the vicious cycle in IBD patients wherein inflammation and thrombosis/coagulation stimulate and intensify each other. It is already established that there is a cross talk between inflammation and thrombosis, in that inflammation can beget thrombosis and thrombosis can beget inflammation. Therefore, platelets offer the unique opportunity to be the target for novel therapeutic strategies in UC and CD, both of which are chronic inflammatory disorders, associated with an increased risk for thrombosis. The overall aim for an anti-inflammatory platelet-based therapy in IBD would be to attenuate platelet activity to limit platelet interactions with leukocytes and endothelial cells, thus reducing the intestinal inflammation and the subsequent risk for thrombosis in distant vascular beds. However, at the same time, it is crucial to preserve the significant role of platelet aggregates as effector cells in the physiological thrombus formation to prevent bleeding complications. Overall, this has lead to the concept that platelet aggregation and platelet activation should be functionally and therapeutically dissociated, with the latter being the desired therapeutic avenue (Danese et al. 2004; Pitchford 2007). Although this seems to be worthwhile, there are multifaceted interferences between platelet activation and aggregation that often complicate this concept.

Current IBD Therapies

There is growing evidence that established IBD drugs might at least in part be therapeutically active by interfering with inflammation-elicited platelet activation and aggregation. The thiol 6-mercaptopurine (and its prodrug azathioprine) has been shown to inhibit human platelet aggregation, and 5-aminosalicylic acid (5-ASA) reduces platelet activation (Thomas et al. 1986; Carty et al. 2000). In addition, the human/mouse chimeric anti-TNF antibody cA2 has been demonstrated to reduce thrombin generation in CD patients, suggesting a possible indirect effect on platelet function (Hommes et al. 1997).

Potential Future Platelet-Specific IBD Therapies

Recent advantages in our understanding of current platelet physiology and their role in UC and CD pathophysiology have led to a variety of novel antiplatelet therapies in human and experimental IBD. Some of these therapies will be discussed in more detail here.

Purinergic Receptors Platelets express three purinergic receptors on their surface: $P2X_1$ (activated by ATP), $P2Y_1$, and $P2Y_{12}$ (both activated by ADP), of which especially the ADP receptors are involved in platelet activation (e.g., P-selectin expression) and aggregation (e.g., PLA formation) (Leon et al. 2003). Clopidogrel and its predecessor, ticlopidine, are the most commonly used platelet purinergic receptor antagonists and are routinely used to inhibit platelet aggregation, especially in the prevention of stroke and myocardial infarction (CAPRIE Steering Committee 1996). In both human and murine models, clopidogrel (a $P2Y_{12}$ antagonist) has shown a promising reduction of platelet-

dependent inflammatory cascades, such as an inhibition of platelet P-selectin expression, a reduction in platelet–leukocyte adhesion, and an attenuated platelet-dependent production of ROS by leukocytes (Evangelista et al. 2005). In addition, clopidogrel has even been successfully tested in a murine model of colitis (Patel et al. 2012). However, its efficacy in human IBD has yet not been proven. In fact, a recent retrospective chart review found no difference in disease progression between IBD patients receiving clopidogrel and a matched IBD control group (Vinod et al. 2012).

P-selectin As mentioned above, P-selectin is expressed on activated platelets as well as endothelial cells and required for aggregation with leukocytes via binding of PSGL-1. Blocking P-selectin or PSGL-1 seems to be desirable in IBD therapy, since it would attenuate the hallmark event of intestinal inflammation-induced trans-endothelial leukocyte recruitment and activation. Using blocking antibodies against P-selectin and PSGL-1, as well as genetically deficient mice, Mori et al. confirmed this concept by showing a profoundly reduced platelet and leukocyte adhesion within the colonic microvasculature in an experimental murine model of colitis following blockade of P-selectin and PSGL-1 (Mori et al. 2005a, b). In human IBD, Ji et al. showed that dermatan sulfate was able to inhibit P-selectin expression on platelets in UC and CD patients (Ji et al. 2004). However, although mechanistically P-selectin seems to be a potential target in IBD therapy, clinical studies testing this hypothesis are still awaiting.

CD40/CD40L The CD40/CD40L signaling pathway is one of the key pathways for the platelet-dependent amplification of inflammation and thrombosis/coagulation and vice versa. Thus, especially CD40L has become a target of interest in modern IBD therapy. In models of experimental colitis, Vowinkel et al. (using the CD40–CD40L pathway inhibitor trapidil), Liu et al. (using an anti-CD40L neutralizing monoclonal antibody), and Gao et al. (using a CD40 antisense oligonucleotide) have established the promising mechanism of blocking CD40–CD40L interactions in intestinal inflammation (Vowinkel et al. 2007; Liu et al. 2000; Gao et al. 2005). In addition, the monoclonal antihuman CD40 antibody ch5D12 has been successfully used in an IBD and shown to be safe and effective for the induction of remission patients with CD (Kasran et al. 2005). Recently, the established IBD drug infliximab (a chimeric monoclonal TNF-α antibody) has been found to reduce circulating plasma levels of platelet-shed sCD40L and prevented CD40 and VCAM-1 upregulation by endothelial cells (Danese et al. 2006).

Thromboxane A_2 TXA_2 is a classical inflammatory mediator of platelet activation. It is predominantly released by activated platelets and is able to further stimulate platelet activation and aggregation. In a murine model of colitis, selective thromboxane synthetase inhibition showed promising results since it reduced inflammatory colonic lesions (Vilaseca et al. 1990). These early experimental results were followed up in human UC and CD patients, as well as in smaller human IBD pilot studies, which confirmed a possible beneficial therapeutic potential (Casellas et al. 1995; Collins and Rampton 1997; Collins et al. 1996; Carty et al. 2000). However, ridogrel (a combined thromboxane synthesis inhibitor and receptor blocker) showed a lack of efficiency when tested in larger independent, randomized, parallel-group trials for UC and CD (Tytgat et al. 2002; Carty et al. 2001). Overall, these results suggest that further investigations into inhibiting TXA_2 as a potential therapeutic target for the treatment of IBD are needed.

Concluding Remarks

Within the last two decades, we have seen a tremendous development in our understanding of the multifaceted roles that platelets play in the pathophysiology of IBD. However, it is also notable that these findings have not yet been fully translated into novel diagnostic or therapeutic tools in the clinical treatment and modern management of IBD. Ongoing research is needed to fully characterize the pro-inflammatory and pro-thrombotic character of platelets in order to be able to target their specific interactions within the mucosal immune system for the treatment of IBD. When we can inhibit the platelet-derived amplification of the chronic intestinal inflammation in UC and CD, we might also be able to shift the balance in the thrombosis/coagulation system to favor a physiological hemostasis, to avoid bleeding complications, and to prevent thrombotic events in remote vascular beds, which until today threatens IBD patients.

Take-Home Messages

- IBD is accompanied a reactive thrombocytosis and platelets in IBD are characterized by an enhanced activation and elevated aggregation.
- Platelets in IBD are primed to form homotypic platelet-platelet and heterotypic platelet-leukocyte aggregates with IBD patients being prone to develop thromboembolic events.
- In IBD, platelets are directly involved in the mucosal immune response and inflammatory cascade.
- Platelets play a role in modulating the intestinal inflammatory phenotype by amplifying inflammation-induced trans-endothelial leukocyte recruitment and activation.

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Platelets and Neurodegenerative Diseases

Lili Donner and Margitta Elvers

Abstract

For decades, platelets have been thought to be an adequate tool for the diagnosis and analysis of neurodegenerative disorders. Although the impact of platelets in Alzheimer's disease (AD) has been extensively studied for many years, the contribution of platelets in other neurological disorders remains an open question. AD is the most common form of senile dementia and is characterized by neurotoxic amyloid-β (Aβ) plaque formation in the brain parenchyma and cerebral blood vessels. However, AD is related to vascular diseases such as stroke and atherosclerosis. Vascular risk factors increase the risk of AD. Moreover, AD patients suffer from cerebrovascular dysfunction with altered blood flow. Platelets are major players in vascular diseases but are also involved in neuroinflammatory diseases such as AD. Platelets express the amyloid precursor protein (APP) and display the complete enzymatic machinery to process APP into Aβ peptides through a signaling pathway known in the brain. Aβ stimulates platelet activation and adhesion, as well as formation of platelet aggregates. AD patients and AD transgenic mice display modified platelet structure and function with alterations in platelet membrane fluidity, generation of reactive oxygen species, altered APP processing, and abnormal platelet activation. Thus, platelets were considered to be an easily accessible biomarker for quick and safe diagnosis of AD. Furthermore, anti-platelet therapy was thought to be effective in the inhibition of AD progression. Clinical trials of the last few years have provided evidence that aspirin, at least, has no beneficial effect on the progression of AD. However, different studies in mice and humans support the hypothesis that platelets not only mirror the disease but actively contribute to the progression of AD.

Introduction

Alzheimer's disease is an age-related neurodegenerative disorder affecting more than 45 million people worldwide (Canobbio et al. 2015). The crucial event in AD is the misfolding and aggregation of amyloid-β (Aβ), leading to the formation of Aβ deposits in brain parenchyma and cerebral vessels (Glenner and Wong 1984; Masters et al. 1985). Recently, it has become evident that AD is a more intriguing disorder that also affects peripheral tissue outside the brain. Alterations in AD patients occur not only in the central nervous system but also in blood vessels and blood cells, with Aβ deposits in cerebral vessels (Thal et al. 2008). Furthermore, AD is strongly related to the vascular system. Vascular risk factors for AD and cerebrovascular dysfunction are observed in AD patients with abnormal blood flow (Iadecola 2004; Luchsinger et al. 2005).

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Alzheimers' Disease: A Neurodegenerative Disorder with High Prevalence

APP Structure and Function

AD is characterized by the accumulation of extracellular plaques containing mostly self-aggregating 40–43 amino acid residue peptides of Aβ and intracellular neurofibrillary tangles (Glenner and Wong 1984; Masters et al. 1985). The Aβ peptides are generated from amyloid precursor protein (APP) (Fig. 1). Human APP belongs to an evolutionarily conserved family of type 1 transmembrane glycoproteins, which also includes the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2) (Goldgaber et al. 1987; Wasco et al. 1993). APP contains a large extracellular N-terminal domain, a transmembrane domain, and a short intracellular cytoplasmic C-terminal domain (Fig. 1). In humans, several alternatively spliced isoforms of APP have been identified (Beyreuther et al. 1993). Whereas the 695 amino acid form is expressed predominantly in neurons, the 751 and 770 amino acid forms containing a Kunitz protease inhibitor domain are ubiquitously expressed in non-neuronal cells (Bayer et al. 1999; Mattson 1997), including platelets (Li et al. 1994). APP is synthesized in the endoplasmic reticulum (ER) and then trafficked through the Golgi apparatus to the trans-Golgi network (TGN) (Xu et al. 1997). The post-translational modifications of APP such as N- and O-linked glycosylation, tyrosine sulfation, and phosphorylation occur in the ER and Golgi/TGN. The modified, mature APP protein can be then shuttled in TGN-derived secretory vesicles to the cell membrane, where most of its cleavage occurs. Afterwards, a small fraction of APP is endocytosed and processed by the endosomal lysosomal pathway (Haass et al. 2012).

The C-terminus of APP can interact with different adaptor proteins, including the G_0 protein (Brouillet et al. 1999), brain proteins Fe65 and X11 (Tomita et al. 1999; Zambrano et al. 1997), JIP (c-Jun N-terminal kinase [Jnk]-interacting protein) protein families (Inomata et al. 2003), and disabled-1 (Dab1) protein (Homayouni et al. 1999). Phosphorylation of APP facilitates interactions with other adaptor proteins such as ShcA and Grb2 via the PTB (phosphotyrosine binding) or SH2 (Src homology 2) domains (Russo et al. 2005). APP can also act as a receptor involved in cell–cell or cell–matrix adhesion processes and can bind to sulfated proteoglycans, laminin, collagen, and integrins (Beher et al. 1996; Ghiso et al. 1992; Small et al. 1992; Williamson et al. 1996).

APP has mainly been studied in relation to AD, but the physiological function of APP is unknown. A number of studies indicate that APP plays an important role in cell growth and proliferation. Moreover, APP influences cell migration, cell adhesion, axonal transport, dendritic outgrowth, and synapse formation (Haass et al. 2012). Studies with mice have shown that APP knockout mice have reduced brain weight, deficient long-term potentiation, and impaired spatial navigation in behavioral tests (Ring et al. 2007). Mice overexpressing APP have enlarged cortical neurons, suggestive of a neurotropic effect (Oh et al. 2009).

APP Processing and Aβ Production

APP can undergo amyloidogenic processing, which occurs mainly at the cell surface, leading to Aβ generation and non-amyloidogenic processing, which takes place in intracellular compartments (Beyreuther et al. 1993; Vetrivel and Thinakaran 2006; Yu et al. 2005; Ling et al. 2003) (Fig. 1). In amyloidogenic processing, cleavage of APP by β-secretase, known as membrane-anchored aspartyl protease BACE (Vassar and Citron 2000), generates the soluble N-terminal fragment APPβ (sAPPβ) and the C-terminal fragment βAPP-CTF. Subsequent cleavage of βAPP-CTF within its transmembrane region by γ-secretase generates Aβ(1-40), Aβ(1-42/43), and APP intracellular domain (AICD) (Haass et al. 2012). In non-amyloidogenic processing, generation of Aβ is precluded because APP is cleaved within the Aβ sequence by α-secretase, which belongs to the metalloproteinase family of Adamlysins including ADAM 10 (Lammich et al. 1999) and ADAM 17 (Buxbaum et al. 1998). Cleavage leads to generation of the soluble, secreted APPα (sAPPα) fragment and the membrane-tethered αAPP-CTF. The remaining αAPP-CTF fragment can be processed by γ-secretase, giving rise to peptide P3 and the APP intracellular domain AICD (Haass et al. 2012) (Fig. 1). The release of AICD into the cytosol may play a role in nuclear signaling (Haass et al. 2012).

Aβ is generated ubiquitously, but clinically relevant Aβ deposition takes place only in the brain. To date, the factors that promote Aβ deposition or prevent its clearance in the brain are not known. Approximately 90 % of secreted Aβ consists of the Aβ(1-40) peptide, whereas only 10 % is Aβ (1-42) under normal conditions. Aβ(1-42) peptides are more amyloidogenic in vitro (Jarrett et al. 1993) and initiate Aβ deposition and plaque formation in vivo because Aβ(1-42) is

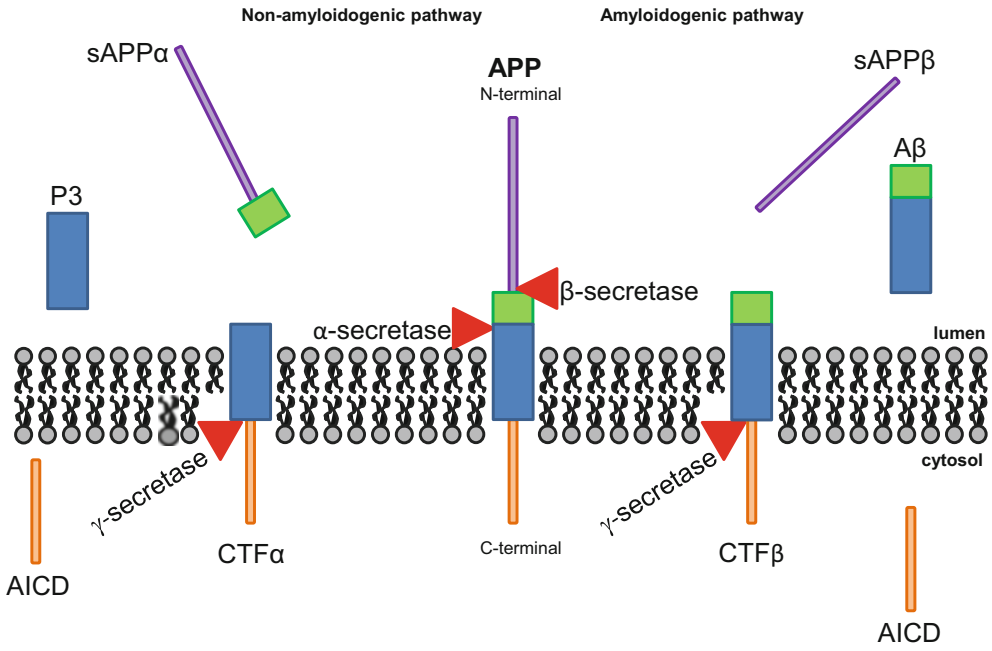

Fig. 1 Proteolytic processing of amyloid precursor protein (APP). APP can undergo non-amyloidogenic (*left*) or amyloidogenic (*right*) processing. Amyloidogenic pathway: APP is first cleaved by β-secretase, resulting in soluble β-APPβ (sAPPβ) and C-terminal β fragment (CTFβ). The cleavage of CTFβ by γ-secretase generates APP intracellular domain (AICD) and Aβ. Non-amyloidogenic pathway: α-Secretase cleaves within the Aβ-domain, generating soluble α-APP fragments (sAPPα) and the C-terminal fragment α (CTFα). The cleavage of CTFα by γ-secretase leads to release of the P3 peptide and AICD

selectively deposited in amyloid plaques (Gravina et al. 1995). Thus, the level of Aβ(1-42) in the brain might play a substantial role in the origin of Aβ deposition.

Cerebral Amyloid Angiopathy

Cerebral amyloid angiopathy (CAA) is characterized by the extracellular deposition of Aβ peptides, mainly Aβ(1-40) (Gravina et al. 1995), in the walls of cortical and leptomeningeal arteries (Glenner and Wong 1984). The incidence of CAA increases with age and occurs sporadically or as a result of genetic mutations (Thal et al. 2008). Risk factors for CAA include mutations of *APP*, presenilin 1 (*PSEN1*, also known as *PS1*) , and presenilin 2 (*PSEN2*, also known as *PS2*) genes and possession of the epsilon 4 allele of apolipoprotein E (Thal et al. 2008). Pathological examination of blood vessels shows loss of smooth muscle cells, luminal narrowing, vessel wall thickening, microaneurysm formation, and intracerebral microhemorrhage (Mandybur 1986; Vinters et al. 1998). CAA is present in over 95 % of AD patients (Jellinger and Attems 2007) and could contribute to neurodegeneration and, thus, to cognitive decline in AD.

Mutation as Risk Factor for Familial AD

About 95 % of AD cases are late onset and sporadic without defined causes, whereas less than 5 % are familial AD, caused by mutations and having an age of onset before 65 years (Zekanowski et al. 2004). Three dominant genes have been identified in cases of familial AD: *APP* on chromosome 21 (Goldgaber et al. 1987; Kang et al. 1987; Tanzi et al. 1987), *PSEN1* on chromosome 14 (Li et al. 1995; Schellenberg and Montine 2012; Tanzi et al. 1987), and *PSEN2* on chromosome 1 (Levy-Lahad et al. 1995). All these mutations have been shown to somehow affect the metabolism of Aβ. Mutations in APP have been identified in a 54 amino acid segment in and around the Aβ peptide sequence, leading to an increase in Aβ formation (Schellenberg and Montine 2012). For example, the Swedish mutation at the N-terminus of the Aβ domain of APP (Mullan et al. 1992) favors β-secretase cleavage (Cai et al. 1993) and is associated with an increase in Aβ peptides in the brain of AD patients. APP23 transgenic mice, which express human APP with only the Swedish mutation, develop parenchymal and cerebrovascular Aβ deposits and have often been used to study CAA pathogenesis (Sturchler-Pierrat et al. 1997). Mutations in the subunits of the γ-secretase enzymatic complex (presenilin 1 or presenilin 2) display alterations in the amino acid sequence of the transmembrane domain that affects the enzymatic processing of APP. Moreover, enhanced production of the less-soluble and more toxic Aβ (1-42) peptide is observed (Schellenberg and Montine 2012).

Platelet APP, Processing and Release of Aβ, Coagulation

Role and Metabolism of APP in Platelets

Platelets contain large amounts of APP, which contributes to more than 90 % of the circulating APP (Chen et al. 1995; Li et al. 1994; Van Nostrand et al. 1990). The concentration of

APP isoforms in platelets is comparable to those in brain but with a different expression pattern. APP695 is the most abundant form in the brain whereas APP770 and APP751 are the predominant forms in platelets (Bush et al. 1990; Li et al. 1995; Selkoe et al. 1988). APP is found at the plasma membrane and in α-granules of platelets (Gowert et al. 2014; Van Nostrand et al. 1990). Full-length APP is expressed on the platelet membrane, encoded by platelet mRNA (Gardella et al. 1990), and may act as a platelet receptor (Li et al. 1994). Platelet activation leads to a threefold increase in surface APP (Li et al. 1994). The physiological role of APP and its metabolites is not yet well understood. Platelets express the complete enzymatic machinery responsible for the metabolism of APP. Under physiological conditions, APP is metabolized through the non-amyloidogenic pathway. α-Secretase is activated by Ca^{2+} and calmodulin (Canobbio et al. 2011) and releases soluble APP (sAPP). Soluble APP dose-dependently inhibits platelet aggregation and secretion in response to stimulation with ADP or adrenaline, suggesting that the release of APP is part of a negative feedback regulation of platelet activation (Henry et al. 1998). Secreted isoforms of APP exhibit the Kunitz proteinase inhibitor domain that is analogous to the cell-secreted proteinase inhibitor known as protease nexin-2. Overexpression of sAPP in circulating platelets of transgenic mice leads to inhibition of thrombosis because of its anticoagulant function, indicating that APP plays an important role in regulating hemostasis and thrombosis (Xu et al. 2009). In fact, sAPP isoforms potently inhibit the intrinsic coagulation factors XIa and IXa (Schmaier et al. 1995; Smith et al. 1990) and this inhibition is enhanced by heparin and zinc (Komiyama et al. 1992).

Processing of APP and Aβ Release

High levels of APP in platelets and the fact that platelets are the major repository of Aβ in blood suggests a direct contribution of platelets to the accumulation of Aβ in brain and in the vasculature (Chen et al. 1995). APP is a transmembrane glycoprotein with a large extracellular N-terminal domain and a short cytoplasmic C-terminal domain that is proteolytically processed in a complex way. Two alternative pathways have been described that involve either α-secretase in the non-amyloidogenic pathway or β-secretase in the amyloidogenic pathway, besides the γ-secretase complex (Kang et al. 1987). Platelets possess α-, β-, and γ-secretases and produce APP fragments comparable to those found in the brain: sAPPα and sAPPβ isoforms, CTFβ amyloidogenic fragment, and Aβ peptides (Li et al. 1998). The α-secretase ADAM-10, the β-secretase BACE1, and the γ-secretase catalytic subunit presenilin were found to be expressed in platelets (Colciaghi et al. 2004; Evin et al. 2003; Vidal et al. 1996). In contrast to neuronal cells, the predominant pathway for APP processing in platelets is cleavage by α-secretase, because high levels of sAPPα are detected but low levels of Aβ (Colciaghi et al. 2004). Moreover, high amounts of sAPPα are stored in platelet granules (Smith and Broze 1992). In contrast to neurons, which produce high amounts of Aβ(1-42) peptides, the Aβ(1-40) peptide is mainly released from platelets. This is in line with observations demonstrating that the circulating Aβ forms contribute to vascular amyloid deposition (CAA) composed mainly of Aβ(1-40), whereas the dominant form in parenchymal amyloid plaques in the brain is Aβ(1-42) (Herzig et al. 2004). In response to platelet activation, full-length APP is proteolytically cleaved by a Ca^{2+}-dependent cysteine protease, providing evidence for the participation of an amyloidogenic species of APP in the proteolytic processing platelets (Chen et al. 2000; Li et al. 1995). In addition, platelets release Aβ upon activation by thrombin, collagen, and synthetic Aβ(1-40), thereby contributing most of the Aβ present in the circulation (Gowert et al. 2014; Li et al. 1998). Different forms of Aβ peptides are processed and released from thrombin-activated and resting platelets with different kinetics, including the classical Aβ peptides, Aβ(1-40) and Aβ(1-42), and several shorter C-terminally truncated forms such as Aβ(1-39), Aβ(1-38), Aβ(1-37), and Aβ(1-34) (Smirnov et al. 2009). The origin of Aβ in blood has been extensively discussed and two major hypotheses have been proposed. Either circulating Aβ derives from the central nervous system via the blood–brain barrier and becomes absorbed by blood cells (mainly by platelets) or additional release of Aβ from non-neuronal cells such as platelets occurs (Di Luca et al. 2000; Prodan et al. 2006).

In atherosclerosis, processing of APP is a mechanism mediated by macrophage activation. The presence of APP, Aβ, and platelets together with inducible NO synthase (iNOS), a marker of macrophage activation, was confirmed in microvessels in advanced human carotid artery plaques (De Meyer et al. 2002). The incubation of human THP-1 macrophages and human platelets induced platelet phagocytosis and the formation of foam cells containing lipids, APP, and Aβ. In contrast, treatment with a protease inhibitor reduced APP processing and inhibited NO biosynthesis. Aβ was present in activated iNOS-expressing perivascular macrophages that had phagocytized platelets, suggesting that platelet-derived APP is proteolytically processed to Aβ, leading to iNOS induction and thus to macrophage activation (De Meyer et al. 2002). Consistently, Aβ peptides promote and synergize chronic inflammatory processes responsible for the degeneration and destruction of arterial vessel walls (Kokjohn et al. 2011). Thus, processing of APP could represent a biochemical link between atherosclerosis and AD; accordingly, nonsteroidal anti-inflammatory drugs (NSAIDs) have an impact in both diseases (Lathe et al. 2014). Interestingly, high levels of Aβ(1-42) were observed

in aged, non-demented individuals with cerebral atherosclerosis but no changes were detected in the β-secretase BACE1 (Sadleir et al. 2013).

Platelet Activation by Aβ

Different studies in the past have shown that Aβ is able to activate platelets, trigger platelet aggregation, and stimulate intracellular signaling pathways (Fig. 2a). Aβ interacts with platelets and supports platelet aggregation via specific platelet membrane receptors in vitro and in vivo (Gowert et al. 2014; Kowalska and Badellino 1994; Wolozin et al. 1998). Activation of platelets by Aβ is mediated at least in part by PLCγ2 and protein kinase C and triggers thrombus formation in vitro and in vivo in venules of mesenteric arterioles (Gowert et al. 2014; Shen et al. 2008a, b). Moreover, preincubation of platelets with Aβ induces activation of the p38 MAPK signaling pathway, including PAR1, PI3K, Akt, and cytosolic phospholipase A2 (cPLA2) activation and thromboxane production (Shen et al. 2008a, b). Platelet adhesion is also supported by Aβ (Barnham et al. 2004; Canobbio et al. 2013; Gowert et al. 2014; Kowalska and Badellino 1994). Immobilized Aβ peptides induce platelet adhesion and spreading and accelerate platelet spreading on collagen under static conditions (Canobbio et al. 2013; Gowert et al. 2014). Under flow conditions, Aβ stimulation enhances platelet adhesion to collagen and, in addition, platelets are able to adhere to immobilized Aβ. In mice, platelet adhesion to the injured carotid artery is enhanced by injection of Aβ-stimulated platelets (Gowert et al. 2014). Adhesion to Aβ peptides results in the activation of Akt, Erk, MLC, and p38 MAPK and induces Rap1b activation (Canobbio et al. 2013). Sonkar and coworkers have shown that Aβ stimulates RhoA activation, leading to modulation of actomyosin organization (Sonkar et al. 2014). Gowert and colleagues found that platelet stimulation with Aβ(1-40) alone does not lead to P-selectin exposure or integrin activation, as measured by flow cytometry. However, Aβ(1-40) increases ADP-stimulated platelet responses and induces phosphatidylserine exposure and platelet microparticle formation (Gowert et al. 2014). In line with these results, Canobbio and colleagues provided evidence for the requirement of ADP for the Aβ-induced signaling pathway, including phosphorylation of Akt, pleckstrin, Erk, p38 MAPK and MLC as well as Rap1b activation and aggregation (Canobbio et al. 2013).

Coagulation: Role of Fibrinogen

In the last few years, a prominent role for fibrin and fibrinolysis in the progression of Aβ pathology has been demonstrated by the group of Sidney Strickland. They observed age-dependent fibrin deposition and increased blood–brain barrier permeability in transgenic mouse models of AD (Paul et al. 2007). The analysis of different transgenic mouse models suggested a direct contribution of fibrin to the pathology of AD. AD mice with only one functional plasminogen gene showed reduced fibrinolysis and increased neurovascular damage compared with AD mice without plasminogen deficiency. Treatment of AD mice with

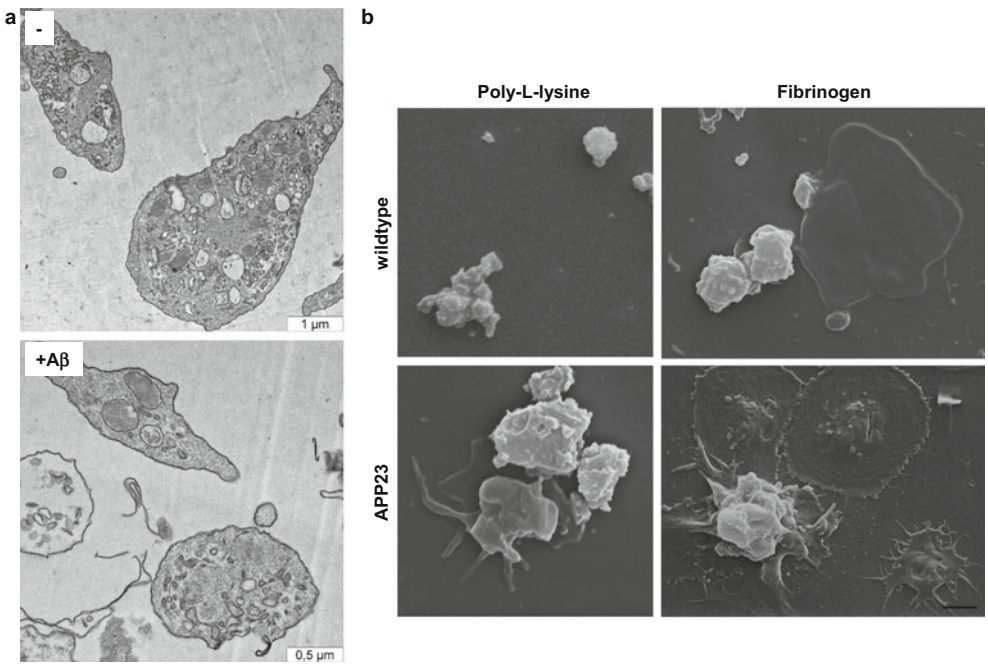

Fig. 2 Platelet activation in response to Aβ. (**a**) Stimulation of platelets with 50 ng/mL Aβ leads to platelet activation, as shown by TEM. Resting platelets (*upper panel*), Aβ stimulated platelets (*lower panel*). *Scale bar* as indicated. (**b**) Representative images show platelets from wild-type and APP23 mice under basal (poly-L-lysine, *left panel*) and stimulating conditions (fibrinogen, *right panel*) as analyzed by scanning electron microscopy (SEM). *Scale bar* 1 µm

the plasmin inhibitor tranexamic acid worsened pathology, and removal of fibrinogen from the circulation of AD mice by ancrod treatment attenuated neuroinflammation and vascular pathology (Paul et al. 2007). Aβ specifically interacts with fibrinogen, inducing its oligomerization (Ahn et al. 2010). The fact that Aβ modifies the structure of fibrinogen implies abnormal fibrin clot formation in AD. Further studies indicated an abnormal resistance to degradation of A-β-modified fibrin clots (Cortes-Canteli et al. 2010). In Aβ-positive blood vessels, fibrinogen was observed in mouse and human AD samples. Intravital brain imaging of clot formation provided evidence for abnormal thrombosis and fibrinolysis in AD mice (Cortes-Canteli et al. 2010). Interestingly, fibrin deposition was increased in AD brains and shown to correlate with the degree of AD pathology (Cortes-Canteli et al. 2015).

Platelets and AD

Altered Structure and Function of Platelets in AD

Data from patients and transgenic mouse models clearly indicate that platelet structure, function, and clearance are altered in AD compared with healthy controls. The most commonly used mouse models of AD are shown in Table 1.

Platelet Membrane Structure and Fluidity

APP processing and Aβ generation occurs within the membrane, therefore the structure and composition of the platelet membrane is important for Aβ production and the progression of AD. Abnormal platelet membrane fluidity in AD has been ascribed to alterations in internal membrane composition but not to differences in membrane phospholipid synthesis (Cohen et al. 1987; Kanof et al. 1991; Swiderek et al. 1997). These changes have never been described for other dementias, for example, multi-infarct dementia (Swiderek et al. 1997). Analysis of the cholesterol content of platelet membranes and red blood cells showed no significant alteration per se; however, a slight decrease in platelet membrane cholesterol was observed in a small group of AD patients. Increased membrane cholesterol was reported to result in increased β-secretase activity, which is important for the generation of Aβ(1-40) (Liu et al. 2009).

Oxidative Stress and Free Radical Generation

Oxidative stress has been implicated in the progression of AD (Barnham et al. 2004). In addition to Aβ-mediated effects on platelet activation, aggregation, adhesion, and thrombus formation, the stimulation of platelets with Aβ induces the generation of reactive oxygen species (ROS) and membrane scrambling (Gowert et al. 2014). ROS overproduction contributes to mitochondrial dysfunction and, thus, to the pathogenesis of AD (Sheehan et al. 1997). ROS levels increase with enhanced environmental stress to control cellular function, including apoptosis (Rada and Leto 2008). Stimulation of platelets with Aβ leads to increased ROS levels with enhanced expression of active caspase-3, which is known to play a central role in cell apoptosis. Caspases also play a role in platelet activation and senescence by promoting the removal of aged or activated platelets from the circulation, suggesting that contact of platelets with Aβ leads to platelet apoptosis (Gowert et al. 2014; Shcherbina and Remold-O'Donnell 1999). Cell shrinkage is another hallmark of cell apoptosis and was observed in platelets stimulated with Aβ, as measured by reduced cell volume, mitochondrial potential, and annexin V binding, thus supporting data showing that Aβ promotes apoptosis of platelets (Gowert et al. 2014).

In contrast to macrophages, platelets are not able to phagocyte synthetic Aβ fibrils or soluble Aβ (Yazawa et al. 2001). However, induction of apoptosis in platelets enables the uptake of pre-aggregated Aβ(1-42) into platelets. Thus, the uptake of Aβ fibrils by apoptotic platelets could be a novel mechanism for local accumulation of Aβ when platelets adhere to vascular amyloid plaques at cerebral vessels and undergo apoptosis (Gowert et al. 2014).

Altered APP Processing

It is well known that platelets of AD patients show AD-specific alterations in APP processing that might reflect abnormal APP metabolism in brain. Colciaghi and coworkers found significant modification of APP,

Table 1 Summary of the most commonly used genetic mouse models of AD

Line	APP23	APP/PS1	3 × Tg-AD	5 × FAD	Tg2576
Amyloid plaques	+	+	+	+	+
Tau-phosphorylation	+	+	+	–	–
Neurofibrillary tangles	–	–	+	–	–
CAA	+	+	n.a.	n.a.	+
Neuronal loss	+	n.a.	+	+	–
Cognitive defects	+	+	+	+	+

n.a. not analyzed/not available

ADAM10, and BACE1 involved in the amyloid cascade, as detected in the earliest clinically detectable state of AD (Colciaghi et al. 2004). This data provides evidence for early metabolic changes towards the amyloidogenic pathway. Abnormalities in the pattern of platelet APP in AD patients have been observed (Bush et al. 1992; Di Luca et al. 1998; Rosenberg et al. 1997). Rosenberg and colleagues measured the ratio between the 120–130 kDa APP isoform and the 110 kDa APP isoform and found that APP processing was affected as compared with healthy subjects (Rosenberg et al. 1997). A correlation between platelet APP isoform ratio and cognition was described by Liu and coworkers, suggesting that a reduction in this ratio could act as a marker of cognitive decline in AD patients (Baskin et al. 2000; Liu et al. 2007). Alterations in APP isoforms show a positive correlation with the progression of clinical symptoms, suggesting that this parameter could be a good marker for determining the progression of AD. Because alterations in APP processing are described as an early event in AD, the APP ratio could have a great diagnostic power and be a very important AD biomarker (Borroni et al. 2002; Di Luca et al. 1998). In contrast to observations with patients suffering from sporadic AD, patients that carry a *PSEN2* mutation have no alteration in the expression or concentration of APP isoforms, suggesting that the pathological effect of this mutation might occur upstream of the amyloid cascade (Pastorino et al. 2000).

Abnormal Platelet Activation

Different studies have provided evidence for altered platelet activation in AD patients and AD transgenic mice. As early as 1998, Sevush and colleagues measured the percentage of circulating platelet aggregates, expression of platelet P-selectin, formation of leukocyte–platelet complexes, and presence of platelet microparticles in AD patients (Sevush et al. 1998). They observed platelet activation under nonstimulating conditions, suggesting that damaged cerebral endothelial cells or membrane abnormalities might be responsible for nonstimulated platelet activation in patients with AD. Stellos and coworkers measured $\alpha_{IIb}\beta_3$ integrin activation and P-selectin exposure for platelets from AD patients under resting conditions. Higher baseline expression of these platelet activation markers were observed in patients with fast cognitive decline compared with patients with slow cognitive decline during a 1-year follow-up period (Stellos et al. 2010). The analysis of cognitive decline in patients with AD revealed that treatment with cilostazol (a phosphodiesterase-3 inhibitor) significantly inhibited changes in cognitive function test scores, whereas patients randomly assigned to clopidogrel or aspirin (platelet inhibitors) showed a statistically significant cognitive decline (Sakurai et al. 2013). Because therapy with platelet inhibitors did not delay cognitive decline, it was speculated that the effect of the phosphodiesterase-3 inhibitor was mediated by platelet inhibition via an increase in cAMP levels or via improvement in endothelial function (Sakurai et al. 2013). However, cilostazol is known for its profoundly unwanted cardiovascular and hemorrhagic properties and is therefore not a useful therapeutic tool for AD patients.

APP23 transgenic mice are known to exhibit vascular and parenchymal Aβ plaques (Winkler et al. 2001), whereas APP Dutch mice show only vascular pathology (Herzig et al. 2004). Analysis of aged APP23 transgenic mice revealed that these mice exhibit pre-activated platelets in the circulation (Fig. 2b). In vitro, platelet activation induced a strong increase in integrin activation and P-selectin exposure compared with age-matched control mice (Jarre et al. 2014). Enhanced platelet activation translated into almost unlimited thrombus formation on collagen under flow ex vivo and accelerated vessel occlusion in vivo. Platelets localized to vascular Aβ deposits in cerebral vessel walls of the AD transgenic mice APP Dutch and APP23. Full occlusion of vessels in APP23 and APP Dutch mice suggests sustained platelet recruitment to vascular Aβ deposits by activated platelets (Gowert et al. 2014) (Fig. 3a). Accordingly, injury of the carotid artery by topical application of $FeCl_3$ led to significantly reduced occlusion times in APP23 mice. Moreover, the infusion of Aβ-stimulated platelets in wild-type mice resulted in enhanced platelet adhesion at the injured carotid artery, suggesting that Aβ-mediated alterations in platelet activation are crucial for cardiovascular and cerebrovascular events in AD (Gowert et al. 2014; Jarre et al. 2014).

Platelets, Coagulation, and Cerebral Amyloid Angiopathy

Different groups have provided strong evidence that platelets are able to process APP and release Aβ upon activation with physiological agonists such as collagen (Smith 1997) and soluble synthetic Aβ(1-40) (Gowert et al. 2014). Aβ(1-40) is the major Aβ peptide released from platelets and is the major component of Aβ deposits in cerebral vessels (Gravina et al. 1995), suggesting that platelets actively contribute to cerebral amyloid angiopathy (CAA).

The reason for increased Aβ accumulation within cerebral vessels is still not well understood. CAA might be the result of altered clearance of Aβ or increased production of Aβ. In 1995, Wisniewski and colleagues observed that smooth muscle cells in the medium supported the deposition of Aβ in the vessel wall (Wisniewski et al. 1995). However, the onset of CAA occurs at the outer basement membrane, suggesting that circulating platelets might trigger CAA. This hypothesis is further supported by the fact that platelets adhere to immobilized Aβ in vitro and to Aβ deposits in cerebral vessels of AD transgenic mice (Fig. 3a).

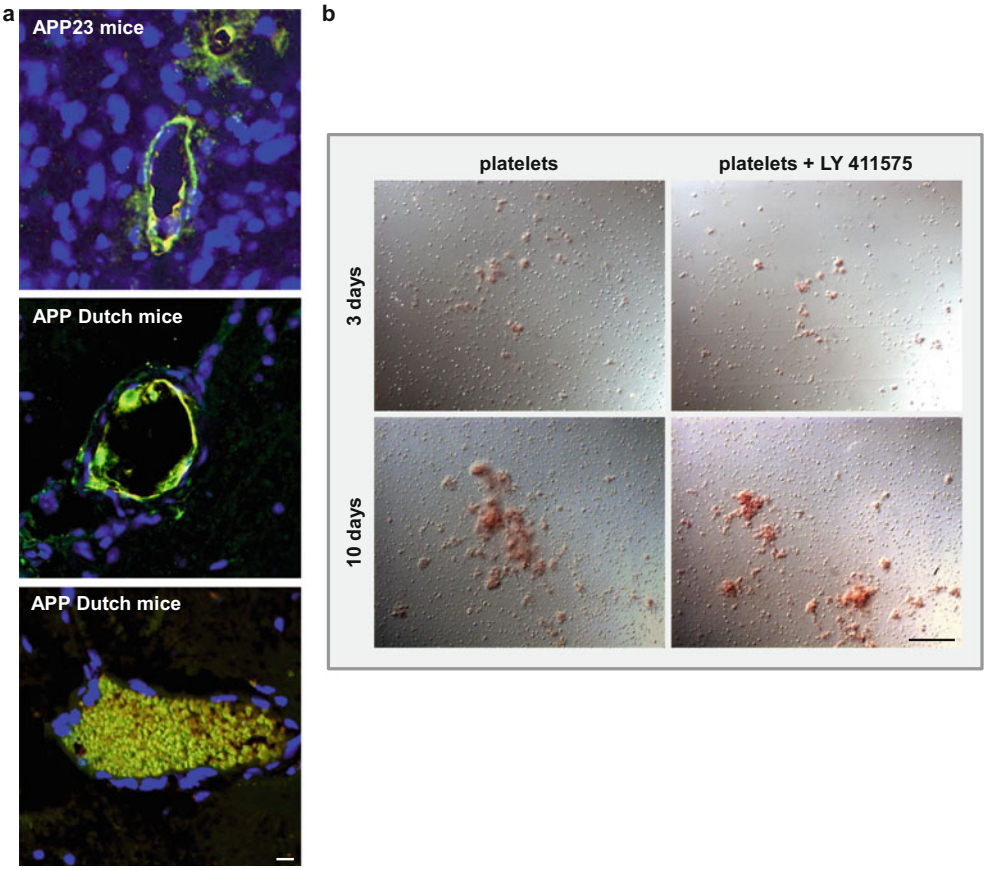

Fig. 3 Platelets adhere to Aβ deposits in cerebral vessels of AD transgenic mice and modulate soluble Aβ into fibrillaraggregates. (**a**) Platelets are recruited to vascular Aβ deposits in cerebral vessels of APP23 (*upper panel*) and APP Dutch mice (*middle* and *lower panels*). Brains were immunohistochemically analyzed for Aβ deposition (6E10, Covance; *red*) and the presence of platelets using the platelet-specific marker GPIb (CD42b, US Biological; *green*). Nuclei staining was performed using 4′,6-diamidine-2-phenylindole (DAPI). Full occlusion of cerebral vessels after sustained platelet recruitment to vascular Aβ deposits (*lower panel*). *Scale bar* 10 μm. (**b**) Congo-Red-positive platelets and Aβ deposits in platelet cell culture following stimulation with 50 μg/mL soluble Aβ after 3 days (*upper panel*) and 10 days (*lower panel*) in culture. To exclude Aβ production by platelets, the γ-secretase inhibitor LY 411575 was used

Interestingly, apoptotic platelets are able to absorb soluble and oligomerized Aβ(1-42), a mechanism that could trigger local accumulation of Aβ at the cerebral vessel wall. Moreover, platelets are able to modulate the conversion of soluble Aβ peptides into fibrillar structures that might also accelerate CAA (Gowert et al. 2014) (Fig. 3b). It is noteworthy that Glanzmann's thrombasthenia (GT) patients whose platelets fail to aggregate in response to stimuli, because they lack or have nonfunctional αIIβ3 integrin, do not suffer from neurodegenerative diseases, suggesting that platelets are markedly involved in the pathology of AD (Nurden et al. 2011).

Studies have confirmed the involvement of fibrinogen in the generation of CAA. Depletion of fibrinogen successfully reduced CAA in AD mice (Cortes-Canteli et al. 2010). Mechanistically, the authors observed oligomerization of fibrinogen in response to Aβ stimulation, which might play a crucial role in AD (Ahn et al. 2010). The newly designed inhibitor RU-505 prevents the Aβ–fibrinogen interaction, leading to restored Aβ-induced alterations in fibrin clot formation and degradation in vitro, and inhibits vessel occlusion in AD mice (Ahn et al. 2014). In a transgenic mouse model of AD (Tg6799 mice), long-term treatment (4 months) with RU-505 resulted in reduced vascular Aβ deposition in the cortex and improved cognitive impairment. Improved neuronal health and attenuated Aβ deposition was also observed with a modest decrease in fibrinogen levels in TgCRND8 mice expressing a double mutant form of APP695, suggesting that blockade of the interaction between fibrinogen and Aβ might be a promising therapeutic strategy in AD (Cortes-Canteli et al. 2015). In AD patients, alteration in the blood–brain barrier could promote the deposition of fibrinogen in cerebral blood vessels and parenchyma and its accumulation in CAA (Paul et al. 2007).

Targeting Platelets in AD

Antiplatelet Therapy

Increasing evidence from studies in AD patients and in AD transgenic mice shows enhanced platelet activation in AD pathology. Thus, anti-platelet therapy might have an effect on the progression of AD. Epidemiological studies in the past suggested that treatment of AD with NSAIDs or aspirin might slow the progression of either vascular or Alzheimer-type pathology. A randomized placebo-controlled clinical trial provided evidence that treatment of AD patients with celecoxib, a selective cyclooxygenase (COX)-2 inhibitor, does not prevent Alzheimer dementia (Lyketsos et al. 2007). A randomized, double-blind study of patients taking rofecoxib, a selective COX-2 inhibitor, also showed that

inhibition of COX-2 is not a useful therapeutic strategy (Thal et al. 2005). In contrast, Broe and colleagues provided evidence for a protective role of anti-inflammatory drugs and aspirin in AD (Broe et al. 2000). However, results obtained from a two-wave longitudinal study over 3.6 years do not support the hypothesis that aspirin or NSAIDs have a protective effect in AD (Henderson et al. 1997).

Aspirin is the most common anticoagulant drug used for the prevention of stroke and myocardial infarction (Yeung and Holinstat 2012). Aspirin irreversibly inhibits COX-1, thus blocking the generation of thromboxane A2 (Loll et al. 1995). High doses of aspirin display anti-inflammatory effects because the production of prostaglandins by white blood cells is inhibited; low doses of aspirin are mainly used for antithrombotic therapy. As shown in the AD2000 randomized open-label trial, treatment of AD patients with aspirin for 2 years has no worthwhile benefit, whereas it increases bleeding risk (Bentham et al. 2008). Cognition and functional ability were evaluated in 156 AD patients who received daily low doses of aspirin and compared with those of 154 AD patients not on aspirin. The results showed no benefit of aspirin therapy on the progression of AD in these subjects, with bleeding complications in 8 % of patients.

Platelets as Biomarker for AD

Peripherial biomarkers play an important role in providing reliable, quick and safe diagnoses. The number of subjects suffering from age-related diseases such as AD is increasing dramatically; therefore, the identification of molecules that can serve as biomarkers is needed. Currently, the definitive diagnosis of AD is possible only post mortem, and the identification of molecular characteristics ante mortem are problematic because of the restricted availability of appropriate sample material. To date, available biomarkers originate from cerebrospinal fluid and are therefore not convenient for routine diagnostics because of the invasiveness of the procedure (Veitinger et al. 2014b). There is a compelling need to develop objective diagnostic and prognostic biomarkers in AD involving easily available tissue, such as blood cells and in particular platelets. Platelets were considered a good model for study of the metabolic changes occurring in AD that might be relevant for the identification of reliable biomarkers (Catricala et al. 2012; Evin and Li 2012; Veitinger et al. 2014b). The alteration in APP processing with altered ratio of APP isoforms (APPr) in AD patients is promising (Evin and Li 2012; Tang et al. 2006; Veitinger et al. 2014b). Determination of APPr might also be relevant for monitoring the outcome of therapies in patients with AD (Evin and Li 2012). Patients with mild to moderate AD were treated with the acetylcholinesterase inhibitor donepezil. After 30 days, an increase in platelet APPr was measured, suggesting that donepezil influences APP metabolism in platelets (Borroni et al. 2002). Zainaghi and colleagues found a decreased ratio of APP130/110 kDa isoforms in patients with AD compared with healthy subjects. Determination of the APPr might also be a promising tool for detection of the conversion from mild cognitive impairment (MCI) to AD, because the baseline levels of the APP130/110 isoform ratio are significantly lower in platelets from patients with MCI who converted to AD compared with patients who remained with stable MCI (Zainaghi et al. 2012). Thus, the measurement of APP isoforms could provide a tool for diagnosis of AD at early stages, for monitoring the progression of AD, and for analysis of a patient's responsiveness to therapeutic intervention (Evin and Li 2012).

Because the balance between non-amyloidogenic and amyloidogenic processing of APP is altered in AD, the sAPPα, ADAM10, and BACE levels in platelets are also promising potential biomarkers (Tang et al. 2006; Veitinger et al. 2014b). Recent studies provide evidence that analysis of platelet activation might have predictive value for the rate of cognitive decline in AD patients (Prodan et al. 2011; Stellos et al. 2010). Significantly higher expression of P-selectin and activated integrin $\alpha_{IIb}\beta_3$ in circulating platelets were observed in patients with AD and fast cognitive decline compared with AD patients showing slow cognitive decline (Stellos et al. 2010). Prodan and coworkers found a relationship between the level of coated platelets and the progression of MCI to AD (Prodan et al. 2011).

In 2014, Veitinger and colleagues presented a platelet protein biochip that can detect an AD specific phenotype (Veitinger et al. 2014a). This array combines protein quantification of monoamine oxidase B and tropomyosin 1 with protein-based genotyping for single nucleotide polymorphisms in the genes encoding apolipoprotein E and glutathione S-transferase omega 1. Although only minimally invasive blood withdrawal is necessary, this biochip enables the identification of AD in patients with a precision of 92 % and could therefore be a promising new tool for the diagnosis of AD.

Interestingly, animal models for studying the molecular mechanisms of AD including platelets are rare, although animal models are known to be a valuable tool in basic and biomedical research. Analysis of brain sections from AD transgenic mice such as APP23 or APP Dutch showed that platelets are recruited to vascular Aβ plaques, where they form aggregates leading to vessel occlusion (Gowert et al. 2014). Increased activation of platelets from AD patients has been described by Stellos and colleagues (Stellos et al. 2010). The analysis of platelet activation in APP23 mice also revealed changes in platelet activation in AD transgenic mice. Activated platelets circulate in these AD transgenic mice (Fig. 2b) and platelet activation with standard platelet agonists induced strongly enhanced platelet activation

compared with age-matched control mice. These results emphasize the need for animal models in the search for new diagnostic tools and to gain more insight into APP–Aβ metabolism in AD (Jarre et al. 2014).

AD and the Risk of Cardio- and Cerebrovascular Diseases

There is a strong relationship between AD, the vascular system, and (cerebro-) vascular diseases such as stroke, atherosclerosis, and hypertension (Catricala et al. 2012; Honig et al. 2003; Mielke et al. 2007). It is now recognized that, on the one hand, vascular risk factors enhance the risk of AD but, on the other hand, AD is related to cerebrovascular dysfunction with blood flow abnormalities that might also interfere with AD pathology (Hardy 2007; Iadecola 2004; Luchsinger et al. 2005; Smith and Greenberg 2009; Staffen et al. 2009). Diabetes and current smoking are the main risk factors; however, hypertension and heart disease can also increase the risk of AD (Luchsinger et al. 2005). Atrial fibrillation, hypertension, and angina are associated with a greater rate of decline and might play a role in secondary prevention of AD (Mielke et al. 2007). The presence of brain infarction influences the course of AD, and increasing evidence suggests that vascular Aβ deposition can impair blood vessel function (Smith and Greenberg 2009). Subcortical infarcts add to the effects of AD pathology by increasing dementia and impaired memory function (Schneider et al. 2007). Importantly, higher platelet activation markers were found in patients with fast cognitive decline compared with patients showing slow cognitive decline. The rate of decline was related to age, platelet activation markers, and a history of myocardial infarction (Stellos et al. 2010).

Vascular Aβ deposition is a common pathology in elderly individuals and is associated with AD. Vascular Aβ is toxic for smooth muscle cells (Mok et al. 2006; Shin et al. 2007) and leads to platelet adhesion and activation, which could induce vessel occlusion, as shown in a transgenic AD mouse model (Gowert et al. 2014) (Fig. 3a). Moreover, toxic Aβ in cerebral vessels causes vascular rupture with intracerebral hemorrhage and reduced blood flow, pointing to a second effect of Aβ in the brain in addition to its direct neurotoxic effects (Smith and Greenberg 2009; Thal et al. 2008). This could represent a mechanism by which CAA is associated with microinfarction and small cortical infarcts. Different studies in the past have analyzed the relationship between vascular function, brain lesions, and clinical symptoms in CAA and AD patients with impaired cognition. Given the fact that more than 90 % of patients with AD display cerebrovascular deposition of Aβ (Attems 2005), studies in mice and humans are needed to determine whether CAA is actively implemented in cognitive decline and is a cause of brain infarction in AD patients. To date, the reason for an increase in Aβ within the cerebral vessel is still unclear. Increased production (Li et al. 2004) or altered clearance of Aβ (Zlokovic 2005) might cause CAA. Moreover, smooth muscle cells in the medium support the deposition of Aβ in the vessel wall (Wisniewski et al. 1995), but the onset of Aβ deposition occurs at the outer basement membrane (Thal et al. 2008). However, platelets adhere to vascular Aβ deposits and might be responsible for the onset of CAA (Fig. 3a). According to the study of Gowert and colleagues, platelet adhesion, activation, and aggregation at Aβ deposits of cerebral vessels might be responsible for complete occlusion of these vessels, as observed in AD transgenic mice known to develop CAA. Moreover, platelets become activated by soluble Aβ (Fig. 2a) and are able to modulate soluble Aβ into fibrillar structures that might contribute to CAA (Gowert et al. 2014). However, the molecular mechanisms and the relation between (cerebro-) vascular pathology and Aβ plaque formation in AD are not yet fully understood. There is a clear indication that cerebral hypoperfusion augments CAA and promotes cortical microinfarcts. In post-mortem human brains, the severity of CAA was a significant predictor of cortical infarcts. Concomitant with human data, AD transgenic mice with CAA showed accelerated vascular Aβ deposition after chronic cerebral hypoperfusion as a result of bilateral carotid artery stenosis (Okamoto et al. 2012). Another study from Garcia-Alloza and colleagues documented that experimental stroke in mice can trigger vascular Aβ deposition and the authors proposed a mechanism that involves interference with amyloid clearance pathways (Garcia-Alloza et al. 2011).

In addition to the association of CAA with cerebral infarcts, the analysis of platelet activation in APP23 mice revealed an enhanced activation profile in these AD transgenic mice compared with age-matched control mice, leading to virtually unlimited thrombus formation under flow ex vivo. In vivo, accelerated vessel occlusion in APP23 mice provided evidence that mice (at least) are at high risk of arterial thrombosis, leading to vascular complications not only in the brain (Jarre et al. 2014). A retrospective study provided evidence for association of Aβ(1-40) levels with progression of arterial stiffness, incident atherosclerosis, and incident coronary heart disease. The study concluded a direct correlation between Aβ(1-40) plasma levels and high risk of cardiovascular mortality in patients with coronary heart disease (Stamatelopoulos et al. 2015). Further studies

are needed to shed light on the relationship between CAA, blood vessels, the risk of cerebro- and cardiovascular complications, and AD.

Other Neurodegenerative Diseases

In the late 1970s, platelets were considered to be an appropriate tool for diagnosis and research in psychiatric and neurologic disorders (Stahl 1977). Platelets can serve as a good model for the transport, metabolism, and release of serotonin (5-HT). It was suggested that the study of 5-HT in blood and platelets might indicate possible abnormalities of 5-HT in neurons of patients suffering from different neurologic diseases such as schizophrenia, Parkinson's disease, and Huntington's chorea. At present, the analysis of platelets and their impact in neurological disorders apart from AD is rare and often descriptive.

Parkinson's disease (PD) is a degenerative disorder of the central nervous system and mainly affects the motor system (Shulman et al. 2011). Mean platelet volume (MPV) was measured in patients with PD and shown to be higher than in AD patients and controls. The authors concluded that MPV could be an indicator of platelet dysfunction (Kocer et al. 2013). To date, platelet activation abnormalities have not been described in PD. Different studies have reported that platelet mitochondrial activity as well as mitochondrial membrane potential in platelets is not altered in PD (Antony et al. 2015).

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with glutamate excitotoxicity (Bos et al. 2006). Platelets were analyzed for alterations in glutamate uptake to assess whether platelets could be a potential peripheral marker for the disease. Platelets from ALS patients showed a significant increase in the expression of glutamine synthetase but normal expression of the glutamate transporter excitatory amino acid transporter 2 (EAAT2). Interestingly, glutamate uptake following thrombin stimulation, as well as under basal conditions, did not differ between platelets from ALS patients and controls (Bos et al. 2006). Dupuis and colleagues analyzed platelet and plasma levels of serotonin and the major serotonin derivate 5-HIAA and found a significant decrease in ALS patients. The levels of platelet serotonin did not correlate with disease duration but were positively correlated with patient survival, suggesting that serotonin influences the progression of ALS (Dupuis et al. 2010).

Huntington's disease (HD) is a neurodegenerative genetic disorder that affects muscle coordination and provokes mental decline and behavioral symptoms (Walker 2007). Several studies have analyzed platelet alterations in HD. In the early 1980s, a few reports described no significant differences in platelet glutamate and aspartate uptake, whereas others found abnormal platelet aggregation in HD compared with controls (Mangano and Schwarcz 1981). In more recent studies, mitochondrial activity was investigated in HD patients. Although normal platelet mitochondrial complex I activity was observed in early HD, when neuronal degeneration is already present (Powers et al. 2007), differences in mitochondrial respiratory chain complex activity and bioenergetic alterations were observed in human platelets before the onset of HD clinical symptoms (Silva et al. 2013). A study from Carrizzo and coworkers provides evidence for nitric oxide dysregulation in platelets from patients with advanced HD, leading to decreased NO levels in platelets from HD subjects (Carrizzo et al. 2014).

Conclusions

For many years, platelets have been deemed a good model for study of the pathogenesis of AD and to serve as a valuable biomarker because they are easily accessible and display the complete machinery for processing Aβ. However, it has become increasingly evident that platelets not only mirror the disease but actively contribute to the progression of AD. Moreover, alterations in platelet activation and thrombus formation suggest that AD patients might be at high risk of cerebro- and cardiovascular complications. Further studies are needed to gain more insight into the molecular mechanisms and the impact of platelets in AD. Our understanding of platelet function and its contribution to other neurodegenerative diseases is still far from complete.

Take Home Messages

- Platelets express the amyloid precursor protein and possess the complete enzymatic machinery for processing amyloid-β
- For decades, platelets were thought to be a valuable model for study of amyloid-β pathology and to serve as a biomarker
- Studies in mice and humans show a strong relation between abnormal platelet activation and generation of reactive oxygen species and the incidence of cerebro- and cardiovascular disease and AD
- Platelets are directly involved in the progression of AD by modulating cerebral amyloid angiopathy
- The impact of platelets with platelet serotonin (5-HT) release, nitric oxide regulation, and mitochondrial activity in psychiatric and neurologic disorders remains an open question

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Platelets and Diabetes

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Abstract

It is increasingly recognized that platelets are the culprit cells implicated in the propensity to atherothrombosis in the setting of both type 1 and type 2 diabetes mellitus, making the study of platelet pathophysiology and platelet activation/inhibition some of the most intriguing fields of research related to diabetes vascular complications. Indeed, a platelet hyperreactive phenotype with biochemical evidence of persistent in vivo platelet activation, with enhanced thromboxane (TX) biosynthesis, has been described in diabetic patients in different stages along the natural history of the impairment of glucose metabolism, even in the preclinical phases.

This chapter highlights several issues within this field: (1) the variable contribution of sustained and acute hyperglycemia, glycemic instability, and insulin resistance to the observed changes in the platelet pathophysiology; (2) the role of oxidative stress and inflammation and namely of platelet-derived inflammatory molecules and microparticles, as secondary mediators of diabetes-induced platelet activation; and (3) the complex intertwining between the described pathophysiology and the anticipated epidemiological burden in terms of high risk of vascular events and lower protection from antithrombotic prophylaxis, with particular reference to aspirin.

Availability of high-throughput techniques is rapidly changing the way we address the problem, with a deeper insight on transcriptomics and posttranscriptional regulation of platelets as a consequence of the above-described metabolic abnormalities.

Abbreviations

ACS	Acute coronary syndrome
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ASA	Aspirin
BMI	Body mass index
CAD	Coronary artery disease
CD40L	CD40 ligand
(COX)-1	Cyclooxygenase
DKK-1	Dickkopf-1
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
MPs	Microparticles
MPV	Mean platelet volume
PAD	Peripheral artery disease
PPG	Postprandial glucose
RAGE	Receptor for advanced glycation end products, AGEs
ROS	Reactive oxygen species
sCD36	Soluble CD36
sCD40L	Soluble CD40L

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T2DM	Type 2 diabetes mellitus
TX	Thromboxane
Wnt	Wingless

Diabetes and Accelerated Atherothrombosis

Type 2 diabetes mellitus (T2DM) is a chronic metabolic disorder characterized by hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism. People with diabetes are at an increased risk of coronary artery disease (CAD), peripheral vascular disease, and cerebrovascular disease (American Diabetes Association 2015a). Although the mortality from CAD in patients without DM has declined since the 1990s, the mortality in men with T2DM has not changed significantly (Roffi et al. 2001). In fact, people with diabetes have a poorer long-term prognosis after myocardial infarction (MI), including an increased risk for congestive heart failure and death.

Diabetes is a strong independent predictor of short- and long-term recurrent ischemic events, in acute coronary syndrome (ACS) (Roffi et al. 2001), including unstable angina and non-ST-elevation MI (Yusuf et al. 2001), ST-elevation MI treated medically (Mak et al. 1997), and ACS undergoing percutaneous coronary intervention (Stuckey et al. 2005). Finally, the concomitant presence of cardiovascular risk factors and comorbidities that negatively affect the outcomes of ACS is higher in this setting (Brogan Jr et al. 2006).

Platelet Activation in Diabetes

Accelerated atherosclerosis in DM may be explained by the fact that the altered metabolic state that accompanies DM is responsible for abnormalities in endothelial and platelet function, which may contribute to the cellular events that cause atherosclerosis and subsequently increase the risk of adverse cardiovascular events.

Alterations in platelet function occur early in the diabetic state and enhanced platelet aggregation predates vessel wall change (Colwell et al. 1983). Moreover, increased platelet aggregation and thromboxane (TX) A_2 synthesis are apparent within days of making rats diabetic with streptozotocin (Gerrard et al. 1980).

Several functional alterations of platelet activity have been observed in DM. Diabetic platelets respond more frequently to subthreshold stimuli, becoming consumed more rapidly, which brings forth to an accelerated thrombopoiesis of more reactive platelets. Thus, platelets from DM subjects are hyperreactive, with enhanced adhesion, aggregation, and thrombin generation (Arjomand et al. 2003), associated with increased platelet generation of thromboxane A_2. Hence, due to such peculiar, disease-related dysregulation of several signaling pathways, leading to a hyperreactive phenotype (Davì and Patrono 2007; Ferreiro et al. 2010), they are regarded as "diabetic" platelets (Fig. 1).

Since 1965, increased platelet aggregation was recognized in DM (Bridges et al. 1965). From then on, augmented platelet degranulation and thromboxane synthesis, mediating further platelet activation, have been demonstrated in DM (D'Angelo et al. 1984). Enhanced platelet activation in DM has also been demonstrated by increased numbers of P-selectin (CD62P)—and CD63-positive platelets, by flow cytometry (Stratmann and Tschoepe 2009). Increased circulating activated platelets can be found even in T1DM, and P-selectin positive platelets can be detected in their metabolically healthy first-degree relatives (Stratmann and Tschoepe 2009). Moreover, thromboxane-dependent platelet activation and endothelial perturbation represent early events in T1DM that are possibly related to an acute inflammatory response (Davì et al. 2003; Romano et al. 2001).

The platelet adenosine diphosphate (ADP) $P2Y_{12}$ receptor signaling pathway is upregulated in diabetic platelets (Matsuno et al. 2005), thus reducing cAMP concentration and enhancing platelet adhesion and aggregation (Ferreira et al. 2006). Moreover, arachidonic acid-stimulated TXA_2 synthesis, determined via its stable metabolite TXB_2, was significantly greater in platelet-rich plasma from diabetic patients than in controls and was positively correlated with the fasting plasma glucose (Matsuno et al. 2005).

Biochemical evidence of increased platelet thromboxane-dependent activation in vivo can be obtained through noninvasive measurements of thromboxane metabolite excretion (Ciabattoni et al. 1989) that avoid artifactual platelet activation during and after blood sampling (FitzGerald et al. 1983). Our group firstly demonstrated that thromboxane A_2 biosynthesis, evaluated in vivo through the urinary excretion of 11-dehydro-TXB_2, is enhanced in patients with T2DM (Davì et al. 1990).

A major issue is whether enhanced platelet activation in DM is merely a consequence of more prevalent atherosclerotic lesions (relevant to the thrombosis risk complicating plaque rupture) or reflects the influence of the accompanying metabolic disturbances on platelet biochemistry and function (Davì et al. 1997). Patients with diabetes and peripheral arterial disease (PAD) had higher TX biosynthesis than age- and sex-matched control subjects (Davì et al. 1990). However, platelet activation has been shown to be rather related to the presence of DM per se, but not to macrovascular disease (Davì et al. 1990). Moreover, alterations in platelet function have been associated with the progression of diabetic microangiopathy, but their role in the prediction of microvascular disease has not been defined (Sobol and Watala 2000; Tarnow et al. 2009). Biochemical evidence

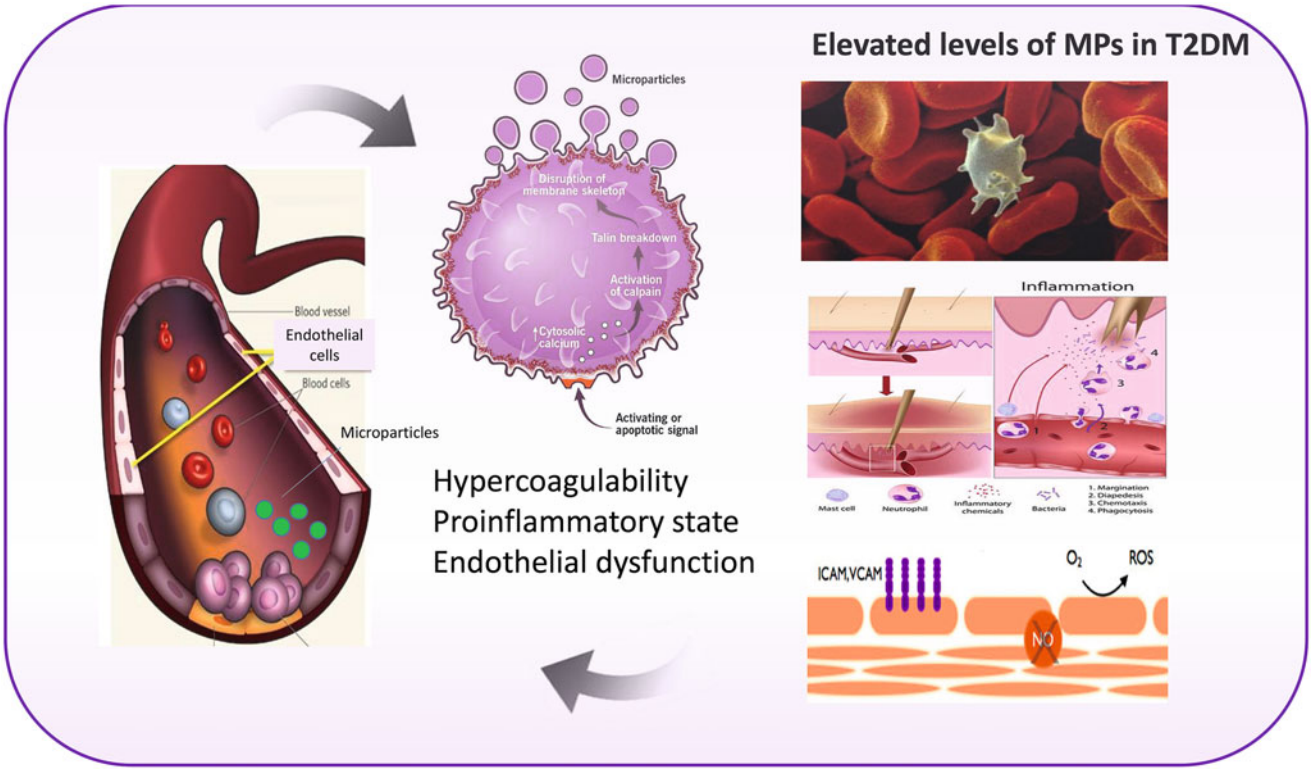

Fig. 1 Metabolic abnormalities in T2DM. The metabolic abnormalities associated with type 2 diabetes may affect platelet transcriptome and/or posttranscriptional regulation through intermediate mediators, such as oxidative stress with isoprostane formation, inflammatory molecule production, endothelial dysfunction with circulating endothelial cells and microparticle release, and cross talk between cells with miRNA exchange through circulating microparticles. The variable contribution and still poorly characterized interaction among these mechanisms eventually result in enhanced platelet hyperreactivity, as reflected by enhanced platelet tissue factor expression and expression of TF-positive platelet–leukocyte complexes; increased expression of activation-dependent adhesion molecules (e.g., $\alpha_{IIb}\beta_3$, P-selectin), reflecting increased aggregability and the cross talk with leukocytes; and increased in vivo arachidonic acid metabolism and increased thromboxane $(TX)A_2$ biosynthesis

of TX-dependent platelet activation in patients with newly diagnosed T2DM, without micro- or macrovascular complications, would support the hypothesis of a primary role of metabolic abnormalities on platelet phenotype (Santilli et al. 2010).

The understanding that platelet activation is not merely the final step of atherothrombosis but takes part in the earlier stages of metabolic diseases, including diabetes, was paralleled by the identification of an additional function for platelets, acting as powerful activators and amplifiers of inflammation and eliciting further platelet activation. Circulating levels of inflammatory mediators derived from platelets, such as soluble CD40 ligand, soluble CD36, Dickkopf-1, and soluble receptor for advanced glycation end products (sRAGE), are higher in patients with diabetes as compared to controls and able to elicit the release of cytokines and chemokines, cell activation, and cell–cell interactions. Their properties make these soluble mediators potential biomarkers of ongoing low-grade inflammation possibly predicting future atherothrombotic events.

Platelets from DM patients may frequently exhibit accelerated turnover, as reflected by the presence of a higher number of reticulated platelets. These circulating cells represent young, mRNA-rich platelets with enhanced proaggregating potential. Reticulated platelets are larger and more reactive platelets, as platelet size correlates with greater platelet reactivity and total release of granular content (Tschoepe et al. 1991). Moreover, larger platelets produce more TXA_2 than smaller platelets. Thus, the increased potential for aggregation of such platelets lowers their threshold for activation, possibly contributing to increased incidence of cardiovascular events in the diabetic setting. Furthermore, these larger platelets of DM patients show a lower response to antiplatelet therapies with aspirin or clopidogrel (Guthikonda et al. 2007, 2008). Hence, mean platelet volume (MPV), a parameter that mirrors in vivo platelet turnover (Bath and Butterworth 1996) and is considered as an independent predictor of vascular events (Vizioli et al. 2009), is increased in diabetes (Hekimsoy et al. 2004), without any correlation between MPV and fasting blood

glucose, HbA1c, and duration of diabetes. MPV increase occurs at the beginning of the disease and persists for its duration, suggesting that the increase in MPV may be due to the diabetic state per se. The mechanisms underlying increased platelet volume in diabetes may be osmotic swelling due to raised levels of some glucose metabolites (Martyn et al. 1986), or, more likely, an increased MPV may reflect higher platelet turnover, as suggested by a high proportion of reticulated platelets found in T2DM (Rocca et al. 2012). It has been suggested that insulin may induce megakaryocytes to produce larger platelets (Watanabe et al. 1987). Whether an increased volume may also mirror functional differences among platelets released by megakaryocytes is still under study. Larger platelets are equipped with a higher content of proteins relevant to hemostasis and thrombosis. Tissue factor (TF), a 47-kDa glycoprotein, is the main activator of the coagulation cascade occurring in physiologic and pathologic conditions (Camera et al. 2003). Platelets can be a source of active TF, the so-called "blood-borne" TF or "circulating" TF, which can sustain activation of the blood coagulation on the edge of a growing thrombus (Camera et al. 2012). Megakaryocytes are committed to release into the circulation a well-characterized number of TF-carrying platelets, representing a fraction of the total platelet population (Camera et al. 2015). If this fraction would coincide with platelets with a larger platelet volume, MPV may also reflect the extent of platelet hyperreactivity and possibly the likelihood to atherothrombosis. Whether TF-containing platelets are more common in the setting of diabetes is currently under deep investigation.

Microparticles (MPs) of platelet origin may promote inflammatory cell recruitment, inducing cell adhesiveness through upregulation of cytokines (RANTES) both in endothelial cells and monocytes (Nomura 2009). MPs may drive vascular inflammation, endothelial dysfunction, leukocyte adhesion, and recruitment (Fig. 2). MPs are increased in a wide range of thrombotic disorders, with an interesting relationship between their levels and disease pathophysiology, activity, or progression. PMPs are also shown to be involved in the progressive formation of atherosclerotic plaque and development of arterial thrombosis, especially in diabetic patients (Santilli et al. 2016). Enhanced levels of TF-bearing MPs have been found in T2DM patients (Ueba et al. 2008). The procoagulant potential of MPs in diabetes (Camera et al. 2003) is increased and related to glycemic control. Thus, several lines of experimental and clinical evidence support a role of MPs as markers of cardiovascular risk and drivers of atherothrombosis in diabetes (Santilli et al. 2016).

This chapter will focus on the strong body of evidence indicating a platelet hyperreactivity phenotype in diabetes, fostered by the variable contribution of a number of mechanisms driven by the metabolic abnormalities associated with T2DM and eventually affecting the platelet transcriptome and/or posttranscriptional regulation. These would include oxidative stress with isoprostane formation, inflammatory molecule production, endothelial dysfunction with circulating endothelial cells and microparticle release, and cross talk between cells with miRNA exchange through circulating microparticles (Fig. 1).

Metabolic Changes and Platelet Activation

Hyperglycemia

The abnormal metabolic state that accompanies diabetes renders arteries susceptible to atherosclerosis, being capable of altering the functional properties of multiple cell types,

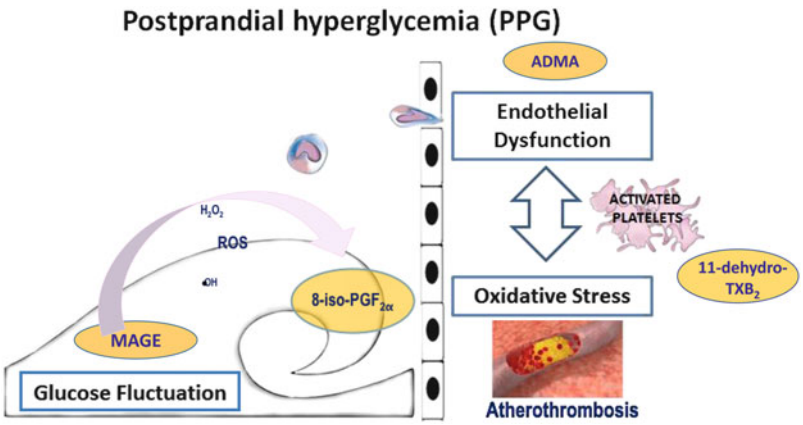

Fig. 2 Microparticles. Platelets are a major source of microvesicles, membrane-coated vesicles that emerge by budding upon apoptosis. Microparticles are both biomarkers and effectors of disease. They expose negatively charged phospholipids, which provide binding sites for activated coagulation factors. Elevated levels of MPs may also promote inflammatory cell recruitment, via their influence on cell–cell interactions and cytokine release. Finally, platelet-derived MPs may promote inflammatory cell recruitment, inducing cell adhesiveness through upregulation of cytokines both in endothelial cells and monocytes

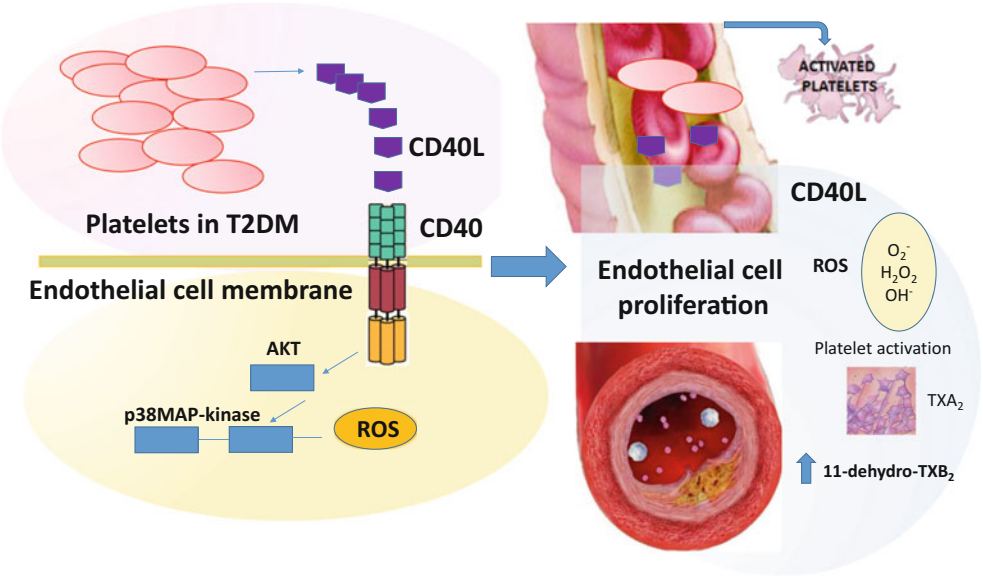

Fig. 3 Glucose fluctuations in diabetes. Glucose postprandial swings exert detrimental effects by increasing oxidative stress and endothelial dysfunction, as mirrored in vivo by circulating levels of asymmetric dimethylarginine (ADMA), with subsequent lipid peroxidation and TX-dependent platelet activation. These abnormalities favor the occurrence of atherothrombosis. Acute glucose fluctuations, as reflected by mean amplitude of glycemic excursions (MAGE), are strongly correlated in vivo with isoprostane generation and thromboxane biosynthesis, as reflected by the urinary 8-iso-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 excretion rates, respectively, both cross-sectionally and during interventions aimed at reducing glucose excursions

including platelets. An altered platelet metabolism and changes in intraplatelet signaling pathways may contribute to the pathogenesis of atherothrombotic complications of diabetes.

A variety of mechanisms may be responsible for enhanced platelet aggregation. Among them, hyperglycemia may represent a causal factor for in vivo platelet activation and may be responsible for nonenzymatic glycation of platelet glycoproteins, causing changes in their structure and conformation, as well as alterations of membrane lipid dynamics. Intraplatelet glucose concentration mirrors the extracellular concentration, since glucose entry into the platelet does not depend on insulin (Vinik et al. 2001). Chronic hyperglycemia upregulates the expression of platelet activation markers (CD31, CD62P, CD63) and platelet surface receptors (GPIb and $\alpha_{IIb}\beta_3$) (Eibl et al. 2004), significantly related to HbA1c levels, suggesting that improvement of metabolic control may be beneficial on platelet activation.

Impaired fasting glucose (IFG)—a prediabetic state—is a frequent glycemic disorder in the general population. MPV is significantly higher both in diabetics and in IFG subjects than in controls (Coban et al. 2006). MPV is positively related to fasting glucose and HbA1c in both diabetics and IFG. This may be a potential mechanism by which subjects with IFG are at increased cardiovascular risk (Santilli et al. 2010).

Postprandial hyperglycemia—an early metabolic abnormality often preceding the clinical diagnosis of diabetes and an independent risk factor for CVD in T2DM (Balkau 2000)—is characterized by postprandial glucose (PPG) deterioration, preceding impairment in fasting glucose level (Cavalot et al. 2006). Thus, impaired glucose tolerance (IGT) reflects the progression from normoglycemia to T2DM. The mechanism(s) by which glucose fluctuations during the postprandial period exert their deleterious effects may include enhanced oxidative stress and endothelial dysfunction (Monnier et al. 2009), both contributing to platelet activation (Fig. 3). Persistent in vivo TX-dependent platelet activation and enhanced lipid peroxidation in response to postprandial hyperglycemic spikes have been demonstrated in patients with early T2DM—with HbA1c levels <7 %—and free of detectable microvascular and macrovascular complications (Coban et al. 2006). Furthermore, only PPG (upward glycemic spikes) predicts TX biosynthesis, consistent with the finding that acute, short-term hyperglycemia, induced by hyperglycemic clamping, enhances thromboxane biosynthesis in T2DM (Ha and Lee 2001). Consistently, other factors, largely reflecting the underlying primary metabolic abnormality, have been reported to predict TX biosynthesis in other clinical settings, such as cholesterol levels in hypercholesterolemia and homocysteine or folate concentration in hyperhomocysteinemia (Davì and Patrono 2007). A moderate decrease in PPG achieved with the α-glucosidase inhibitor acarbose causes time-dependent downregulation of both urinary TX metabolite

excretion, suggesting a causal link between early metabolic abnormalities and platelet activation in this setting (Balkau 2000). A recent study from our group, comparing the extent of TX-dependent platelet activation in IGT, DM patients with a diagnosis documented either <12 months or ≥ 12 months, demonstrated that thromboxane biosynthesis—at study entry—was comparably enhanced in the three groups, with limited intra-subject variability over time (Ciaffardini et al. 2014).

Insulin Resistance

T2DM is frequently associated with insulin resistance and, consequently, with increased levels of circulating insulin, especially at the beginning of the natural history of diabetes.

Insulin resistance or its associated hyperinsulinemia are independent risk factors for CAD, with a level of risk similar to that of hyperlipidemia (Despres et al. 1996). Increased insulin resistance rather than decreased insulin secretion are related to inflammation (Blake and Ridker 2001) even in the prediabetic state (Festa et al. 2003). Insulin resistance per se contributes to increased platelet activation, independently of underlying inflammation. Platelets retain a functional insulin receptor capable of insulin binding and autophosphorylation (Falcon et al. 1988). After receptor binding, insulin inhibits $P2Y_{12}$ signaling and thereby reduces platelet reactivity (Ferreira et al. 2004). Thus, insulin is generally thought to reduce platelet responses to various agonists (Abrahm et al. 1986). Insulin also increases the surface expression of PGI_2 receptors, thus exerting an antiplatelet action by maintaining platelet sensitivity to PGI_2 (Abrahm et al. 1986). Decreased platelet insulin receptor number and affinity (Udvardy et al. 1985) might account for platelet hyperactivity in T2DM. Alteration of insulin action enables platelet dysfunction (Kahn 1998).

Insulin resistance increases intraplatelet calcium concentration that leads to augmented degranulation and aggregation. Moreover, insulin resistance reduces platelet sensitivity to NO and PGI_2, both released by the endothelium with an inhibitory effect on platelet function (Nissen et al. 2008).

Thus, the finding that human platelets have insulin receptors that participate in the regulation of platelet function (Falcon et al. 1988) led to the hypothesis that in clinical settings characterized by decreased insulin sensitivity, platelets are potential sites of insulin resistance and that the latter is associated with impairment in the physiological antiaggregating action exerted by insulin (Trovati and Anfossi 2002). Whereas insulin inhibits platelet aggregation in healthy non-obese subjects, this effect is blunted in obese individuals (Trovati et al. 1995; Monnier et al. 2006). Thus, an impaired platelet response to insulin could be another feature of the insulin resistance syndrome.

Oxidative Stress

DM is strictly linked with oxidative stress, with overproduction of reactive oxygen species (ROS), as well as with reduced antioxidant levels, in both type 1 (Chiarelli et al. 2004, 2005) and type 2 diabetes (Jardin et al. 2006; Davì et al. 2010). Enhanced generation of oxidants, such as superoxide anions and hydrogen peroxide, is able to increase platelet activation (Freedman 2008).

Hyperglycemic spikes may advise ROS production directly via glucose metabolism and auto-oxidation and indirectly through the formation of AGE and their receptor binding. ROS, in turn, may activate other signaling molecules, such as PKC and NF-kB, leading to transcription of redox-sensitive genes (Gresele et al. 2003). These glycated proteins may contribute to the development of atherosclerotic complications by activation of the receptor for AGEs (RAGE) (Santilli et al. 2009a). Due to increased ROS production, plasma from patients whose diabetes is poorly controlled has less antioxidant capacity (Tsai et al. 1994) and contains increased levels of lipid hydroperoxides (Nourooz-Zadeh et al. 1995) and F_2-isoprostanes, such as 8-iso-prostaglandin (PG) $F_{2\alpha}$ (Davì et al. 1999), only partially reversible in association with improved glycemic control. 8-iso-$PGF_{2\alpha}$ is considered a nonenzymatic oxidation product of arachidonic acid in circulating LDL and is a marker of lipid peroxidation both in vitro and in vivo (Ciaffardini et al. 2014).

Increased urinary excretion of 8-iso-$PGF_{2\alpha}$ has been depicted in association with diabetes (Patrono and FitzGerald 1997). Moreover, improvement of metabolic setting and vitamin E supplementation in T2DM patients are both accompanied by a significant reduction in the urinary excretion of 8-iso-$PGF_{2\alpha}$ and of 11-dehydro-TXB_2 (a stable metabolite of TXB_2) (Blake and Ridker 2001; Formoso et al. 2008). Thus, changes in the rate of arachidonate peroxidation to form biologically active isoeicosanoids, such as 8-iso-$PGF_{2\alpha}$, may represent an important biochemical link between altered glycemic control, oxidant stress, and platelet activation in T2DM (Blake and Ridker 2001).

Of interest, increased lipid peroxidation and platelet activation denote early events in the development of T1DM subjects (Davì et al. 2003). Subjects with newly diagnosed diabetes had significantly increased urinary excretion of both 8-iso-$PGF_{2\alpha}$ and 11-dehydro-TXB_2 and higher plasma levels of a number of inflammatory markers such as IL-6 and TNF-α. They were reduced after 1 year from the onset of diabetes. Thus, biochemical signals of oxidative stress and platelet activation can be appreciated early at the onset of diabetes, and their variable intensity is driven, at least in part, by IL-6 production and disease duration (Davì et al. 2003).

Moreover, both lipid and protein oxidation are significantly elevated in patients with T2DM (De Cristofaro et al. 2003). The association of F_2-isoprostane formation with both urinary 11-dehydro-TXB_2 excretion and plasma prothrombin fragment F1 + 2 levels argues that lipid peroxidation can influence platelet as well as coagulative activation, thus contributing to a prothrombotic state in T2DM (Falcon et al. 1988). Oxidized lipids put on activated platelets may give a better surface for the assembly and activation of prothrombinase complex. This may promote a vicious cycle that, together with the oxidation linked depression of the anticoagulant pathway, may predispose to a prothrombotic state in diabetes mellitus (Davì et al. 2002).

Lastly, oxidative modifications of circulating proteins have been involved in the increased platelet hyperactivity seen in diabetic patients. Glycoxidation of albumin, with glycated albumin generation, blunts the nonesterified fatty acid-fixing ability of albumin, thus supporting enhanced arachidonate oxygenation made eligible for the production of TXA_2 (Blache et al. 2015).

Several pathways are involved in hyperglycemia-induced vascular damages: (1) enhanced polyol activity, causing sorbitol and fructose accumulation; (2) increased formation of advanced glycation end products (AGEs); (3) activation of protein kinase C and nuclear factor kB; and (4) increased hexosamine pathway flux (Brownlee 2001). Hyperglycemia triggers these deleterious metabolic events through a single process: overproduction of superoxide by the mitochondrial electron-transport chain.

Glucose swings reveal a triggering effect on oxidative stress (Monnier et al. 2006). In the postprandial phase, glucose variations induce oxidative stress more than chronic hyperglycemia. The MAGE, obtained by measuring the arithmetic mean of the differences between consecutive peaks and nadirs (Service et al. 1970), is used for assessing glucose fluctuations during the day, as a marker of glycemic instability (Service et al. 1987; Tschoepe et al. 1991). Acute glucose fluctuations are strongly correlated with isoprostane generation, while no relationship was observed when urinary 8-iso-$PGF_{2\alpha}$ excretion rates were plotted against main markers of sustained hyperglycemia (HbA1c and mean daily glucose concentrations) (Basili et al. 2006).

Diabetes and Platelet-Derived Inflammation

Platelets represent an important linkage between inflammation, thrombosis, and atherogenesis. Platelet-induced chronic inflammatory processes at the vascular wall result in development of atherosclerotic lesions and atherothrombosis (May et al. 2007).

Hyperglycemia, glycemic variability, and insulin resistance are determinants and predictors of platelet activation and consequently also of the release of inflammatory mediators such as CD40 ligand, CD36, Dickkopf-1, and presumably soluble receptor for advanced glycation end products (sRAGE), which amplify the inflammatory status further promoting the release of cytokines and chemokines.

CD40 signaling mediates many inflammatory responses in atherosclerosis. A wide variety of inflammatory cells express CD40 ligand (**CD40L**), and stimulation by other pro-inflammatory cytokines increases endothelial cell expression of CD40L (Fig. 4). Upregulation of CD40L at baseline prospectively predicts cardiovascular events among apparently healthy women. Elevated soluble CD40L (sCD40L) levels have been found in both type 1 and 2 diabetes, further supporting the idea of a common basis for both diabetes and atherosclerosis (Santilli et al. 2006).

Soluble **CD36** (sCD36) has been proposed to early identify diabetics at risk of accelerated atherothrombosis. CD36 is believed to play a critical role in the initiation of atherosclerotic lesions through its ability to bind and internalize modified LDL trapped in the arterial wall, facilitating the formation of lipid-engorged macrophage foam cells (Libby 2002). Additionally, CD36 has been proposed as a marker of macrophage activation and inflammation (Tuomisto et al. 2005). Thus, monocyte CD36 seems to be upregulated under conditions that follow T2DM and the metabolic syndrome (Nakata et al. 1999). Platelet CD36 is associated with nonreceptor tyrosine kinases of the Src family (Huang et al. 1991) implicated in platelet activation by ox-LDL. LDL cholesterol can be oxidatively modified by monocytes via the MPO-H_2O_2-NO_2 system, to form ligands with high affinity for CD36 ($oxPC_{CD36}$). $oxPC_{CD36}$ interaction with platelet CD36 results in increased platelet activation and enhanced thrombus formation at sites of vascular injury in vivo (Jackson and Calkin 2007). Increased plasma CD36 levels have been described not only in overt T2DM (Koonen et al. 2011) but also in prediabetic conditions such as polycystic ovary syndrome and in patients with IGT (Glintborg et al. 2008). sCD36 levels may be predicted by chronic low-grade inflammatory state (CRP and IL-6), low insulin sensitivity, and higher BMI (Handberg et al. 2009) all involved in the pathogenesis of DM and metabolic syndrome. We have provided in vivo evidence that persistently increased levels of sCD36 in T2DM patients are related to impaired metabolic control and to diabetes duration (Liani et al. 2012). Furthermore, we have observed a highly significant correlation between sCD36 and the urinary excretion rate of 11-dehydro-TxB_2, supporting the likelihood of CD36 release during TxA_2-dependent platelet activation in T2DM. Interestingly, a linear and direct correlation has been reported between plasma CD36 levels and the urinary excretion rate of 8-iso-$PGF_{2\alpha}$, marker of in vivo ongoing lipid peroxidation (Patrono et al. 2005) related to circulating ox-LDL levels (Liu et al. 2004). This is in agreement with

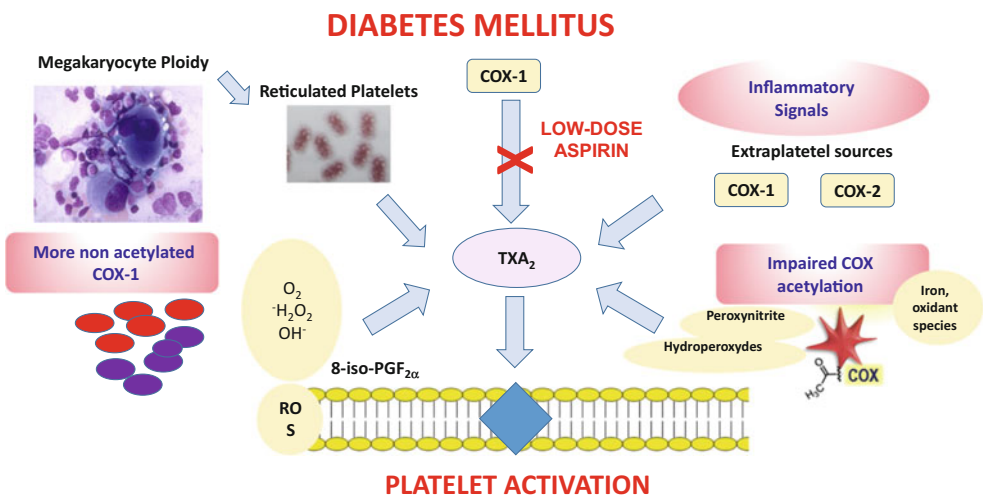

Fig. 4 CD40 signaling. CD40L is a 39-kD transmembrane glycoprotein structurally related to tumor necrosis factor (TNF)-α. More than 95 % of the circulating CD40L derives from platelets. This mediator is stored in the cytoplasm of resting platelets, exposed on the surface upon platelet activation, cleaved, and released into the extracellular environment. This product of cleavage, soluble CD40L, in turn induces inflammatory responses in the endothelium by binding CD40 on endothelial cells. CD40L also increases platelet release of ROS through activation of Akt and p38 MAP kinase signaling pathways. The linear correlation between plasma CD40L levels and the urinary excretion rate of the enzymatic metabolite of TXB_2, 11-dehydro-TXB_2, supports the likelihood of CD40L release during TXA_2-dependent platelet activation in T2DM

the evidence that a specific CD36-dependent, ox-LDL-mediated signaling pathway is required for platelet activation (Chen et al. 2008).

Dickkopf-1 (DKK-1) is the pivotal regulator of the wingless (Wnt) signaling pathway, involved in inflammation, atherogenesis, and control of glucose metabolism (Chien and Moon 2011). Wnt signaling modulates atherosclerosis, regulating endothelial function, vascular smooth muscle cell proliferation, angiogenesis, and inflammation, mediating both inflammatory and anti-inflammatory effects. Wnt pathways are directed by multiple antagonists or modulators including dickkopfs (DKK). Platelets are major sources of circulating levels of DKK-1 in several clinical settings (Lattanzio et al. 2014). Based on the evidence of enhanced DKK-1 expression in platelets (Voorzanger-Rousselot et al. 2009), we supposed that diabetic subjects with higher platelet activation in vivo may exhibit increased circulating DKK-1. Thereafter, we speculated that the increased concentrations of DKK-1 in T2DM may derive, at least in part, from TX-dependent platelet activation. Thus, for the first time, we reported that in diabetic patients enhanced circulating levels of the Wnt antagonist DKK-1 are deeply associated with 11-dehydro-TXB_2, ADMA, and CD40L levels. Indeed, DKK-1 levels in T2DM may mirror interactions between inflammation, endothelial dysfunction, and platelet activation (Voorzanger-Rousselot et al. 2009). As a matter of fact, we revealed in vivo that in T2DM patients, DKK-1 is enhanced since the earlier stages of the disease (Vazzana et al. 2012), and amelioration of glycemic control, exerted for instance by acarbose, downregulates plasma DKK-1 levels. Such considerable reduction of DKK-1 after glycemic control improvement and the positive link between changes in the two parameters suggest that hyperglycemia may be a significant driver of soluble DKK-1. In addition, the detected downregulation of plasma DKK-1 in low-dose ASA-treated subjects vs untreated patients makes platelets a likely source of its release (Aukrust et al. 1999).

RAGE (receptor for advanced glycation end products, AGEs) initiates the intracellular signaling that disrupts the cellular function through its recognition and binding of AGEs (Goldin et al. 2006). AGEs contribute to several microvascular and macrovascular complications through formation of cross-links between molecules in the basement membrane of extracellular matrix. AGEs are prevalent in the diabetic vasculature and contribute to the development of atherosclerosis. Soluble forms of RAGE (sRAGE), including the splice variant endogenous secretory (esRAGE), have been found in blood and tissues (Schmidt et al. 2001). Ligand engagement of RAGE triggers cell-specific signaling, resulting in enhanced generation of ROS and activation of NF-kB (Hasan et al. 2003). By occupying the ligand-binding domain, sRAGE might act as an endogenous competitive inhibitor of RAGE, thus influencing the modulatory network of the ligand–RAGE axis (Santilli et al. 2009a) that plays a critical role in the interplay between hyperglycemia and vascular homeostasis (Basta et al. 2006). We described lower plasma sRAGE in subjects with T2DM as compared to nondiabetic subjects, with a significant inverse correlation between circulating sRAGE and HbA_{1c}, insulin resistance

index, S100A12, and CRP. Moreover, in diabetic patients, in vivo oxidative stress and endothelial dysfunction were associated with low sRAGE, as reflected by urinary 8-iso-$PGF_{2\alpha}$ and plasma asymmetric dimethylarginine (ADMA), respectively (Devangelio et al. 2007). These findings suggest that the ligand–RAGE hyperactivity, supported by poor metabolic control, increases in vivo ROS generation, thus promoting systemic inflammation, endothelial dysfunction, and platelet activation (Ferreiro et al. 2010). AGEs strongly activate platelets, increasing RAGE expression at the platelet surface membrane (Gawlowski et al. 2009). As in diabetes, sRAGE levels are reduced in hypercholesterolemic patients and are inversely correlated with oxidative stress and endothelial dysfunction (Yilmaz et al. 2009). Regardless of the underlying metabolic abnormality (familial combined hyperlipidemia or metabolic syndrome), circulating esRAGE discriminates also patients with NAFLD, suggesting that activation of the AGE/RAGE pathway may contribute to the progression of both liver and cardiovascular disease (Santilli et al. 2015a).

Despite no formal demonstration that platelets are a source of soluble RAGE, this hypothesis is suggested by indirect in vivo data, showing that low endogenous secretory RAGE (esRAGE) levels are significant predictors of urinary 11-dehydro-TXB_2 excretion rate in several clinical settings (Santilli et al. 2015a).

Aspirin in T2DM

Platelets are primary targets of antithrombotic therapy in diabetic cardiovascular disease (Jennings 2009).

Aspirin (**ASA**) exerts its platelet action mainly by irreversibly acetylating a serine residue of platelet cyclooxygenase (COX)-1 (Roth et al. 1975), thus inhibiting the formation of thromboxane (TX) A_2. This effect is irreversible because platelets are enucleate and, thus, unable to resynthesize COX-1.

Aspirin is the pivotal agent for secondary prevention among patients with diabetes (Kumbhani et al. 2015) significantly reducing the risk for ACS, stroke, and cardiovascular death (Kumbhani and Bhatt 2010). ADA recommends the use of low-dose ASA (75–162 mg/day) for secondary prevention of cerebrovascular and cardiovascular events in all diabetic patients with history of CVD (American Diabetes Association 2015b).

In primary prevention, the most recent ACCF/AHA guidelines recommend low-dose ASA in people who are at increased CVD risk (10-year risk of CVD events over 10 %) and who are not at increased risk of bleeding, whereas ASA is not recommended for diabetic subjects with low CVD risk (10-year CVD risk under 5 %) as the potential adverse effects from bleeding offset the potential benefits. ADA recommends ASA therapy (75–162 mg/day) as a primary prevention strategy at increased cardiovascular risk such as men aged >50 years or women aged >60 years who have at least one additional major risk factor (family history of CVD, hypertension, smoking, dyslipidemia, or albuminuria) (American Diabetes Association 2015b). Ongoing trials will provide further insights into the appropriateness of ASA prescription in the primary prevention setting in diabetic subjects. These include A Study of Cardiovascular Events iN Diabetes (ASCEND) and Aspirin and Simvastatin Combination for Cardiovascular Events Prevention Trial in Diabetes (ACCEPT-D).

ASA Resistance or Hyporesponsiveness?

Among the different antiplatelet strategies, over the past years, the term "aspirin resistance" has been largely abused and inappropriately applied to define both the occurrence of vascular events despite ongoing treatment (clinical definition) and the residual platelet reactivity or impaired platelet response in aspirin-treated individuals (laboratory definition). While clinical failure is an anticipated event for any preventive strategy, given the multifactorial nature of atherothrombosis, laboratory failure is often biased by the variable accuracy of methods employed to assess the response to aspirin.

Although largely inappropriate, the term aspirin resistance has prospered for long, in particular in the setting of diabetes, driven both by the pathophysiological evidence of enhanced platelet hyperreactivity/activation in this setting and by the epidemiological evidence of suboptimal response to aspirin in patients with diabetes, as emerged in metanalyses of clinical trials in this setting (Pignone et al. 2010). Heterogeneity among evaluated studies, low event rate, different aspirin regimens tested, and high rate of aspirin discontinuation may have biased the available results.

While acknowledging the multiple limitations related to the epidemiological evidence fostering the aspirin failure hypothesis, there is compelling pathophysiological evidence for a residual platelet hyperreactivity, variably assessed among studies, despite ongoing low-dose aspirin treatment, at least in a fraction of diabetic patients, leading to a slow shift from the term "aspirin resistance" into "variability of aspirin response."

In this regard, a number of questions arise, whose solution may help in substantiating and providing clinical relevance to the whole story: first, whether methods used to assess platelet inhibition by aspirin are accurate and reliable;

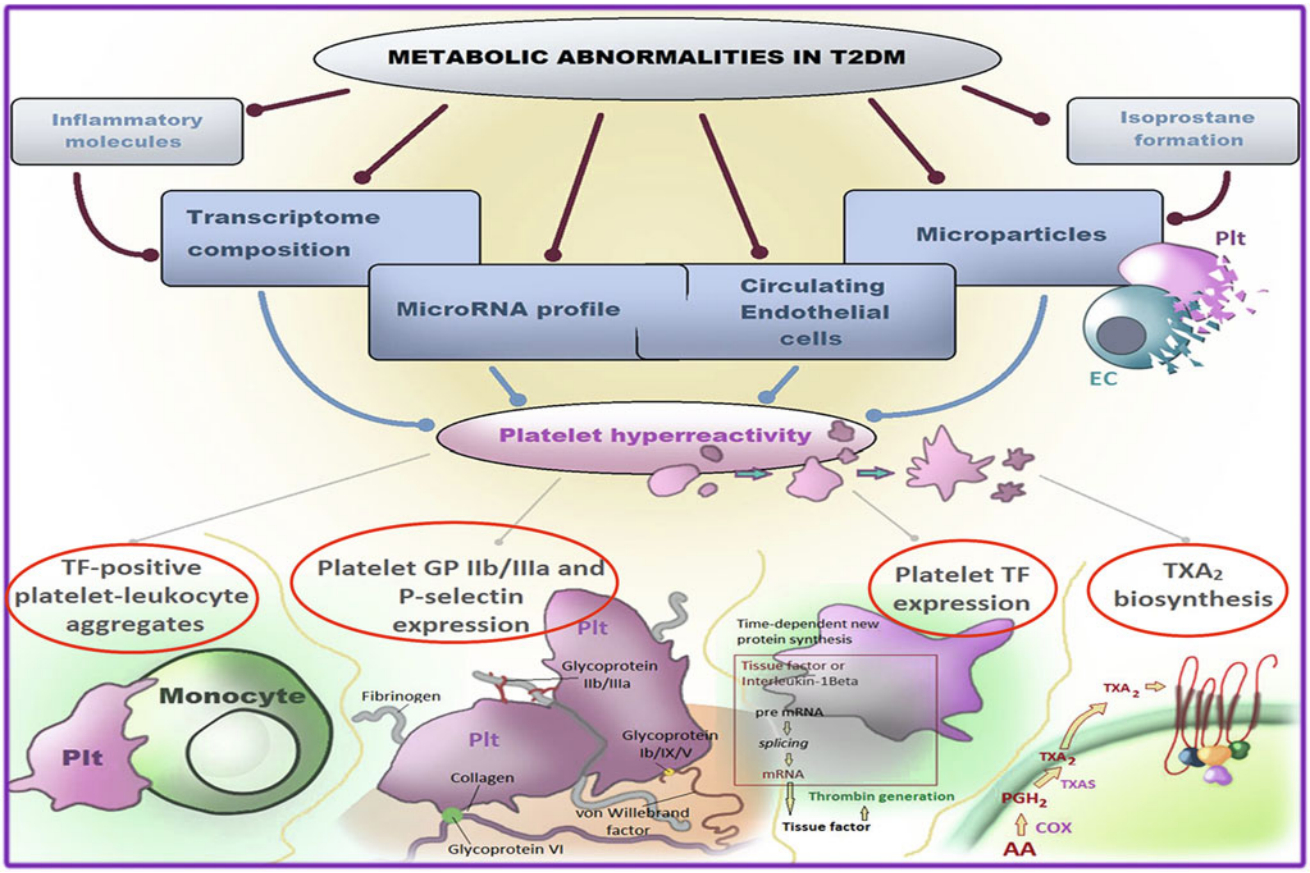

Fig. 5 Less-than expected response to ASA. Diabetic patients may have a lower response to aspirin probably due to an increased megakaryocyte ploidy and accelerated platelet turnover, with an excess of newly released, non-acetylated COX-1 over the 24-h dosing interval that is not inhibited by the once-daily low-dose ASA prescription; low-grade inflammation with increased TXA_2 generation by extraplatelet sources of COX-1 and COX-2; oxidative stress, with increasing reactive oxygen species (ROS) production, nonenzymatic lipid peroxidation leading to formation of biologically active compounds, such as 8-iso-$PGF_{2\alpha}$, able to bind TP receptor, thus contributing to TX-dependent platelet activation; and impaired COX-1 inhibition, by a number of mechanisms, the most likely in this clinical setting being enhanced lipid hydroperoxide ambient

second, whether there is evidence that the presence of diabetes may modulate the inhibitory effect of low-dose aspirin on platelet function; and third, which, if any, easily detectable clinical or biochemical characteristics may identify individuals who would gain inadequate response to aspirin.

Serum TXB_2 and urinary 11-dehydro-TXB_2 are biochemical endpoints targeting aspirin mechanism of action and provide reliable information on the maximal biosynthetic capacity of circulating platelets ex vivo and on the actual rate of TXA_2 biosynthesis in vivo, respectively (Davì and Patrono 2007). In contrast, while platelet COX-1, as reflected by serum TXB_2 levels, is uniformly and persistently suppressed by ASA treatment, the effect of ASA is variably detected by functional assays, potentially leading to misclassification of "responder" as "resistant" phenotypes owing to poor reproducibility of functional measurements on repeated measurements (Santilli et al. 2008, 2009b).

In addition to common causes of suboptimal response to aspirin, such as poor compliance, pharmacodynamic interaction with NSAIDs, as well as pharmacokinetic issues such as prescription of enteric-coated formulations of aspirin, the evidence that the inhibitory effect of low-dose aspirin on platelet function may be modified by the presence of diabetes is mounting.

Excess fat, which is largely prevalent in the diabetic population, may affect aspirin pharmacokinetics due to a larger distribution volume. Adequate bioavailability may be restored by weight loss and/or avoidance of enteric-coated formulations.

In diabetes, lower response to aspirin may be related to (a) enhanced platelet turnover, through increased reactivity of younger platelets; (b) increased TXA_2 formation by platelet COX-2 expression or extraplatelet, cellular COX-1, and/or COX-2, during low-grade inflammation; and (c) incomplete inhibition of COX-1 (Fig. 5). Furthermore, oxidative stress, characterizing diabetes, may favor the escape of platelets from aspirin effects, with multiple mechanisms (Santilli et al. 2015b):

1. Mechanisms impairing the effects of endogenous antiaggregant agents, i.e., reduced platelet sensitivity to the antiaggregating effects of nitric oxide, due to high glucose-mediated oxidative stress
2. Mechanisms interfering with platelet COX acetylation, e.g., COX-1 polymorphisms (Pettinella et al. 2009) and lipid hydroperoxide-dependent impaired acetylating effects of aspirin
3. Mechanisms favoring platelet priming (lipid hydroperoxides) or activation (F2-isoprostanes, acting as partial agonists of thromboxane receptor) or aldose-reductase pathway-mediated oxidative stress, leading to enhanced platelet TXA_2 biosynthesis or TX receptor activation.
4. Mechanisms favoring platelet recruitment, promoted by aspirin-triggered platelet isoprostane formation (Cangemi et al. 2012).

Among all described mechanisms, one of the most convincing is attributable to increased platelet turnover. Unacetylated COX-1 and COX-2 in newly generated platelets may provide a possible explanation for ASA-insensitive TXA_2 biosynthesis: one daily dose of low-dose ASA may be inadequate because of the large capability of bone marrow to accelerate platelet production, even tenfold of basal rate. Several circulating platelets entering the bloodstream after ASA washout will have their COX-1 activity uninhibited and may be responsible for accelerated recovery of TX generation in the 12–24-h dosing interval. Several groups, including ours, have reported that a twice-daily dosing in diabetic patients may overcome this issue (Rocca/Santilli/Pitocco et al. 2012). In our diabetic population (Rocca/Santilli/Pitocco et al. 2012), the shorter duration of COX-1 suppression in about one-third of the studied population was unrelated to glycemic control or diabetes duration, but was likely to be due to abnormal megakaryopoiesis, obesity, or both. Indeed, on multiple regression analysis, higher MPV and body mass index (BMI) were the only independent predictors of accelerated COX-1 recovery. Thus, BMI and MPV may be regarded as candidate and easily measured biomarkers to be tested in larger population, to identify DM patients who may achieve less antithrombotic protection by aspirin and for whom personalized dosing regimens may be tested to overcome interindividual variability.

Conclusions

It is apparent, we would say, lapalissian, that platelets, despite being small, anucleated cells, are the major players and address the propensity to atherothrombosis of the patients with diabetes mellitus.

More recent evidence shows that platelets have the capacity of protein synthesis through translation of megakaryocyte-derived mRNAs and may change their mRNA/protein composition and thus their functions, upon stimulation and/or disease. Thus, in the last years, availability of high-throughput techniques is allowing testing the hypothesis that the metabolic abnormalities associated with T2DM may affect PLT transcriptome and/or posttranscriptional regulation through intermediate mediators, such as oxidative stress with isoprostane formation, inflammatory molecule production, endothelial dysfunction with release of circulating endothelial cell or microparticles, and cross talk between cells with miRNA exchange through circulating microparticles (Fig. 1). Such "molecularly (re)programmed" platelets might influence the development and progression of atherothrombosis in diabetes. Concurrently, molecules produced by platelets will have the potential to serve as biomarkers of the thrombotic risk or the response to antiplatelet treatment.

Take Home Messages

- Platelets are the culprit cells implicated in the propensity to atherothrombosis in the setting of both type 1 and type 2 diabetes mellitus, and are related to diabetes vascular complications.
- A persistent in vivo platelet activation, with enhanced thromboxane (TX) biosynthesis, has been described in diabetic patients, even in the preclinical phases of the natural history of the disease.
- Acute hyperglycemia, glycemic instability, and insulin resistance affect platelet pathophysiology.
- Platelet-derived inflammatory molecules, markers of oxidative stress and inflammation, are secondary mediators of diabetes-induced platelet activation.
- Molecules produced by platelets have the potential to serve as biomarkers of the thrombotic risk or the response to antiplatelet treatment.
- The metabolic abnormalities associated with type 2 diabetes may affect platelet transcriptome and/or posttranscriptional regulation through intermediate mediators, such as oxidative stress with isoprostane formation, inflammatory molecule production, endothelial dysfunction with circulating endothelial cells and microparticles release, cross-talk between cells with miRNA exchange through circulating microparticles.

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Part VI

Pharmacology

Aspirin

Carlo Patrono

Abstract

The last 35 years of clinical research on aspirin have proven its value as a lifesaving drug for the treatment and prevention of atherothrombosis. The place of low-dose aspirin in the therapeutic armamentarium has not been substantially altered by the successful development of new antiplatelet agents, including $P2Y_{12}$ and PAR-1 receptor blockers. The demonstration of additive beneficial effects resulting from effective blockade of the platelet ADP and/or thrombin receptor combined with thromboxane suppression in high-risk patients is consistent with the multifactorial nature of atherothrombosis. Besides becoming an essential component of the antithrombotic strategy in high-risk settings, low-dose aspirin has also provided a mechanistic insight into the participation of platelets in other pathophysiologic processes, including colorectal cancer. The aim of this chapter is to review the mechanism of action and clinical pharmacology of aspirin as an antiplatelet agent, as well as the randomized trial evidence supporting its efficacy and safety. Moreover, more recent findings on additional long-term benefits of aspirin therapy will be discussed and new developments put into perspective.

Historical Perspective

Although synthesized in an industrial environment in 1897 and marketed in 1899, acetylsalicylic acid (aspirin) remains a cornerstone of antiplatelet therapy for the treatment of acute ischemic syndromes, both coronary and cerebrovascular, as well as for their secondary prevention (Amsterdam et al. 2014; Lansberg et al. 2012; Parekh et al. 2013; Roffi et al. 2016; Vandvik et al. 2012). In contrast, its role in the primary prevention of atherothrombosis has been the subject of debate during the past 10 years and remains highly controversial (Patrono 2013). Historically, aspirin has evolved from a prototypic nonsteroidal anti-inflammatory drug (NSAID) to a highly targeted antiplatelet drug (Born and Patrono 2006), largely as a consequence of fundamental discoveries on its mechanism of action in the early seventies (Vane 1971; Hamberg et al. 1975; Roth et al. 1975) (Fig. 1). These include the discovery by J. Vane (1971) that aspirin inhibits prostaglandin (PG) synthesis, the discovery by M. Hamberg and B. Samuelsson (1975) of thromboxane (TX) A_2 as the major arachidonic acid (AA) metabolite in human platelets responsible for the pro-aggregating effect of AA, and the characterization by P. Majerus and his colleagues (1975) of acetylation of platelet PG-synthase as the molecular mechanism responsible for permanent inactivation of its cyclooxygenase (COX) activity. These pivotal studies provided the basis for the development of mechanism-based biomarkers of TXA_2 inhibition in man, i.e., serum TXB_2 (Patrono et al. 1980) and urinary TX metabolite (TXM) excretion (Roberts et al. 1981). The availability of these biomarkers allowed characterizing the dose and time dependence of the inhibitory effects of aspirin on platelet TXA_2 production in healthy subjects (Patrignani et al. 1982; FitzGerald et al. 1983). The consistent demonstration of saturability of the platelet-inhibiting effect of

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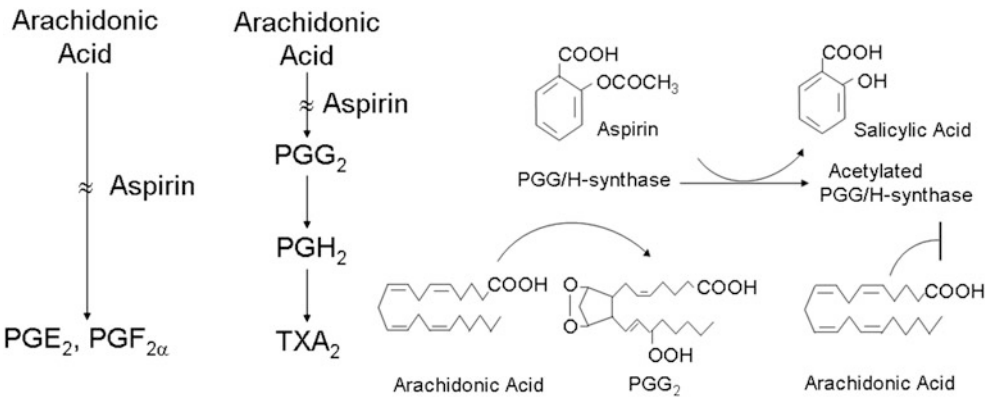

Fig. 1 Fundamental discoveries on the mechanism of action of aspirin. The figure illustrates the discovery by J. Vane (1971) that aspirin inhibits prostaglandin (PG) synthesis; the discovery by B. Samuelsson's Group (1975) of thromboxane (TX) A_2 as the major arachidonic acid (AA) metabolite in human platelets responsible for the pro-aggregating effect of AA; and the characterization by P. Majerus' Group (1975) of acetylation of platelet PG-synthase as the molecular mechanism responsible for permanent inactivation of its cyclooxygenase (COX) activity

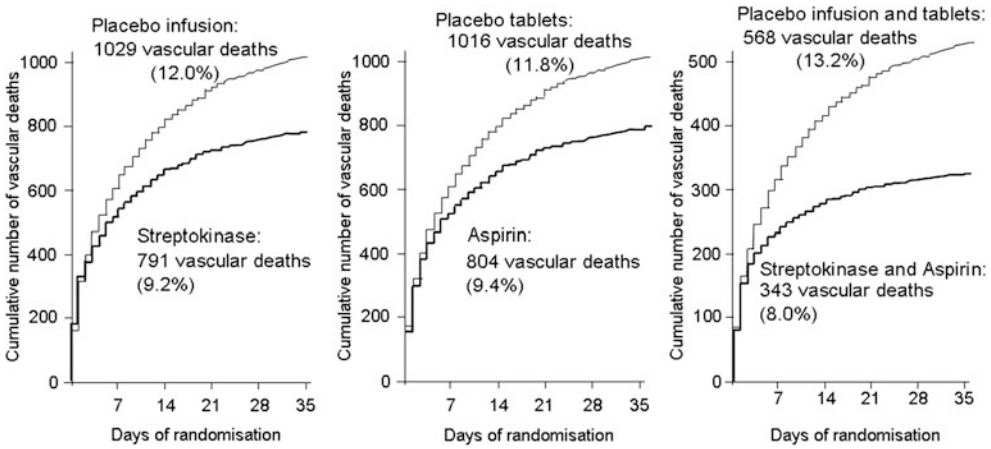

Fig. 2 Effects of streptokinase, low-dose aspirin, both or neither on 5-week vascular mortality in 17,187 patients with suspected acute myocardial infarction. Reproduced from ISIS-2 Collaborative Group (1988) with permission from The Lancet

aspirin at low doses given once daily and the characterization of its biochemical selectivity in sparing prostacyclin (PGI_2) biosynthesis (Patrignani et al. 1982; FitzGerald et al. 1983) provided the rationale for testing the clinical efficacy and safety of a low-dose aspirin regimen in a variety of high cardiovascular (CV) risk settings (Patrono 1994) including acute myocardial infarction (MI) (ISIS-2-Collaborative Group, 1988). The publication of the International Study of Infarct Survival (ISIS)-2 (1988), demonstrating a remarkable protective effect of low-dose aspirin against vascular mortality at 5 weeks after a suspected acute MI (Fig. 2), represented the turning point for the use of aspirin as an antithrombotic agent and provided perhaps the most convincing evidence for an important role of platelet TXA_2 in the pathophysiology of coronary atherothrombosis (Davì and Patrono 2007).

Pharmacokinetics

Aspirin is rapidly absorbed in the stomach and upper small intestine, primarily by passive diffusion of non-dissociated acetylsalicylic acid across gastrointestinal (GI) membranes. Drug plasma levels peak at 30–40 min after the ingestion of uncoated aspirin (Pedersen and FitzGerald 1984). In contrast, it can take up to 4 or 5 h for plasma aspirin levels to peak after the administration of enteric-coated formulations (Patrignani et al. 2014). Orally administered aspirin undergoes substantial presystemic hydrolysis to salicylic acid in the gut content, the gut wall, and the liver (Pedersen and FitzGerald 1984). The systemic bioavailability of regular aspirin tablets is approximately 45–50 % over a wide range of doses (20–1300 mg) (Pedersen and FitzGerald

1984). However, lower bioavailability of enteric-coated preparations due to poor absorption from the higher pH environment of the small intestine may result in inadequate platelet inhibition (Cox et al. 2006; Grosser et al. 2013), particularly in obese subjects (Cox et al. 2006). Aspirin first comes into contact with platelets in the gut capillaries during absorption, and, as a consequence, platelets are exposed to higher drug levels than are present in the systemic circulation (Pedersen and FitzGerald 1984). This may explain, at least in part, the relative biochemical selectivity of low-dose aspirin (75–100 mg once daily) in sparing vascular PGI_2 production (Pedersen and FitzGerald 1984).

Aspirin has a half-life of 15–20 min in the bloodstream (Pedersen and FitzGerald 1984; Patrignani et al. 2014). Despite the rapid clearance of aspirin from the circulation, its antiplatelet effect lasts for the platelet life-span (8–10 days) owing to the permanent inactivation of platelet PGH-synthase (PGHS)-1, an effect that can be reversed only through the generation of new platelets. Given the short half-life of aspirin in the systemic circulation, the long-lasting duration of its antiplatelet effect is ensured by acetylation of PGHS-1 in the bone marrow megakaryocytes and limited new protein synthesis in anucleate platelets. Thus, there is a complete dissociation between the pharmacokinetics and pharmacodynamics of aspirin, allowing the use of a once-a-day regimen for antiplatelet therapy. However, reduced systemic bioavailability of the drug or faster renewal of the drug target may reduce the duration of its full antiplatelet effect and require a different (e.g., twice daily) dosing regimen (Rocca et al. 2012; Pascale et al. 2012) (see below).

Mechanism of Action

Aspirin inhibits platelet function through a molecularly sophisticated mechanism of action that appears ideally suited to target anucleate cell fragments, i.e., by rapidly inducing an irreversible modification in a critical platelet protein that cannot be repaired during the 24-h dosing interval. The limited systemic bioavailability of the active moiety, acetylsalicylic acid, and its short half-life contribute to restraining the extent and duration of any extra-platelet effects of the drug.

Aspirin exerts its unique antiplatelet effects by selectively acetylating a single serine residue (Ser-529) of PGHS-1 (DeWitt et al. 1990). This causes permanent inactivation of the COX activity of the enzyme, which catalyzes the conversion of AA to PGG_2, but does not appreciably affect its peroxidase activity responsible for reducing PGG_2 to PGH_2. Aspirin inhibits the COX activity of PGHS-1 by placing a larger than normal side chain at position 529 thereby interfering with arachidonate binding to the COX active site (DeWitt et al. 1990). Acetylation of the PGHS-1 and PGHS-2 by aspirin is regulated by the catalytic activity of the peroxidase which yields a higher oxidative state of the enzyme (Bala et al. 2008). In cells with high levels of hydroperoxy-fatty acids, the efficacy of aspirin in acetylating PGHS-2 is markedly decreased as compared to cells with low levels of hydroperoxides (Bala et al. 2008). This finding may explain, at least in part, the differential dose requirements for the anti-inflammatory effects (largely dependent on PGHS-2 inhibition, as with other NSAIDs) as compared to the antiplatelet effects of aspirin (entirely dependent on PGHS-1 inhibition), despite comparable potency in inhibiting the two purified enzymes in vitro (Bala et al. 2008). Presystemic acetylation of platelet PGHS-1 (Pedersen and FitzGerald 1984), as noted above, as well as the cumulative nature of inactivation of platelet COX-1 activity upon repeated daily dosing (Patrignani et al. 1982) may also contribute to the relative biochemical selectivity of low-dose aspirin in vivo (Patrignani et al. 1982; FitzGerald et al. 1983).

PGHS-1 and PGHS-2 are homodimers composed of identical subunits, but it has been shown that only one subunit is active at a time during catalysis; moreover, many NSAIDs bind to a single subunit of a PGHS dimer to inhibit the COX activity of the entire dimer (Shimokawa and Smith 1992). However, 50 % acetylation of platelet PGHS-1 is not sufficient to fully suppress the maximal capacity to synthesize TXA_2, and partial acetylation of the second subunit is required in order to suppress platelet COX-1 activity completely (Patrignani et al. 2014; Li et al. 2014).

Permanent inactivation of platelet COX-1 by low-dose aspirin represents the best characterized mechanism of action to explain its clinical efficacy as an antithrombotic agent (Patrono 1994). The finding that the acetylation of platelet PGHS-1 (Patrignani et al. 2014), the inhibition of TXA2 production (Patrignani et al. 1982), and the clinical efficacy of aspirin in reducing the risk of major vascular events in high-risk patients (ATT Collaboration 2002) are saturable at low doses (i.e., even much higher doses are not more effective) allows establishing a cause-effect relationship between this selective acetylation process and its clinical readout (s) (Patrono 2015). Although aspirin can acetylate a number of plasma proteins (e.g., fibrinogen or prothrombin), enzymes, and DNA in vitro (Patrono 2015), this usually requires millimolar concentrations that are approximately 100–1000 times higher than those achievable after oral dosing of low-dose aspirin (Patrignani et al. 2014). Thus, the apparently heterogeneous therapeutic effects of low-dose aspirin may well reflect the multifaceted consequences of platelet COX-1 inhibition on pathophysiological processes (e.g., response to injury and tissue repair) that participate in such diverse diseases as coronary atherothrombosis and colorectal cancer, rather than "pleiotropic" effects on different drug targets (Patrono 2015).

Pharmacodynamics

The pharmacodynamics of aspirin as an antiplatelet agent can be adequately described by serum TXB_2 measurements (Patrono et al. 1980, 1985; Patrignani et al. 1982). Platelet TXA_2 production during whole blood clotting, as reflected by serum TXB_2, represents a highly specific and sensitive index of the maximal biosynthetic capacity of circulating platelets in response to endogenously formed thrombin (Patrono et al. 1980). This biomarker is ideally suited to characterize the pharmacological inhibition of platelet COX-1 activity, when the inhibitor is added in vitro or administered in vivo (Patrono et al. 1980; Patrignani et al. 1982). After oral dosing of aspirin in healthy subjects, serum TXB_2 concentrations are reduced in a dose-dependent fashion with a maximal inhibitory effect achieved after a single dose of 100 mg (Patrono et al. 1980; Patrignani et al. 1982). However, because of the cumulative nature of platelet COX-1 inactivation upon repeated daily dosing (Patrignani et al. 1982), the same ceiling effect of virtually 100 % suppression of serum TXB_2 can be obtained with daily doses as low as 30 mg (Patrignani et al. 1982). Earlier studies using the ability of ingested aspirin to inhibit subsequent in vitro acetylation of PGHS-1 in washed platelets by [^{3}H-acetyl]-aspirin (Burch et al. 1978) underestimated the potency of aspirin in inhibiting COX-1 activity because full acetylation of the enzyme is not required in order to inhibit completely TXA_2 production (Patrignani et al. 2014; Li et al. 2014). Burch et al (1978) reported that a daily dose of 325 mg was required to inhibit by >95 % subsequent acetylation of the platelet enzyme by [^{3}H-acetyl]-aspirin but acknowledged that "the degree to which cyclooxygenase must be inhibited to alter thrombosis is unknown." Similarly, in the study of FitzGerald et al (1983), urinary TXM excretion was maximally inhibited at doses of 325 mg daily and above, most likely because extraplatelet sources contribute appreciably to TXM excretion and are less sensitive to the inhibitory effect of aspirin.

Upon discontinuing aspirin after a brief course of 325 mg per day, no new enzyme appeared in circulating platelets for approximately 2 days (Burch et al. 1978). The authors interpreted the "lag" in the return of unacetylated enzyme to the circulation as evidence that aspirin acetylates PGHS-1 in the bone marrow megakaryocyte (Burch et al. 1978). In fact, human megakaryocytes express both PGHS-1 and PGHS-2 (Rocca et al. 2002). Similarly, Patrignani et al (1982) observed a 2-day lag in the recovery of serum TXB_2 following discontinuation of a 30-day regimen of 30 mg daily. Thereafter, both the unacetylated enzyme (Burch et al. 1978) and serum TXB_2 (Patrignani et al. 1982) returned toward pre-aspirin levels with a linear time course consistent with platelet turnover (life-span 8.2±2 days). Therefore, bone marrow megakaryocytes represent an important drug target contributing to the long-lasting antiplatelet effect of aspirin, inasmuch as the new platelets released during 24–48 h after dosing most likely carry PGHS-1 that has been acetylated in their progenitors. However, substantial interindividual variability in the rate of recovery of platelet COX-1 activity has been described in aspirin-treated patients with vascular disease (Rocca et al. 2012). Accelerated platelet turnover, as seen in association with type 2 diabetes mellitus (T2DM) and following coronary artery bypass graft (CABG) surgery, as well as reduced systemic bioavailability of enteric-coated aspirin in obese subjects, may shorten the duration of the full inhibitory effect on platelet TXA_2 production to less than 24 h and require more frequent dosing (Rocca et al. 2012; Pascale et al. 2012; Patrono et al. 2013; Paikin et al. 2015) (Fig. 3). Variable duration of the antiplatelet effect of aspirin, noncompliance, or a pharmacodynamic interaction with other NSAIDs (see below) may all be responsible for less than complete inhibition of serum TXB_2 and TXA_2-dependent platelet function measured 24 h after dosing. However, these phenomena should not be erroneously labeled as aspirin "resistance," because they all have a plausible explanation and can be easily diagnosed and corrected (Patrono and Rocca 2007; Grosser et al. 2013). If "resistance" is defined in classical pharmacological terms, i.e., as the failure of actual aspirin intake to fully inactivate platelet COX-1 (Patrono and Rocca 2007), then aspirin resistance is either a very rare phenomenon (Santilli et al. 2009; Grosser et al. 2013) or does not exist. A relatively large study of 400 healthy volunteers failed to identify a single case of true drug resistance (Grosser et al. 2013).

Because of its unique pharmacokinetic and pharmacodynamic features, aspirin has a lesser inhibitory effect on PGI_2 than on TXA_2 biosynthesis at all doses, reaching a ceiling effect on the former at 650–1300 mg daily (FitzGerald et al. 1983). Substantial sparing of in vivo PGI_2 production, as reflected by PGI_2 metabolite (PGIM) excretion, has been reported during administration of aspirin 100 mg daily both in health (Capone et al. 2004) and disease (Cavalca et al. 2014). It is likely that substantial inhibition of PGI_2 biosynthesis at higher aspirin doses reflects dose-dependent acetylation of vascular (both endothelial and smooth muscle cell) PGHS-2 (McAdam et al. 1999; Yu et al. 2012). The clinical significance of a PGI_2-sparing regimen of aspirin administration remained largely hypothetical for about 25 years after the original proposal of endothelium-derived PGI_2 representing an important mediator of vessel-wall thromboresistance (Moncada and Vane 1979). The clinical development of the coxibs as selective inhibitors of COX-2 (FitzGerald and Patrono 2001) and their placebo-controlled, long-term trials have provided convincing evidence that inhibition of vascular PGI_2 biosynthesis unaccompanied by adequate inhibition of platelet TXA_2 production is associated with a doubling of the risk of major coronary events (Kearney et al. 2006; CNT

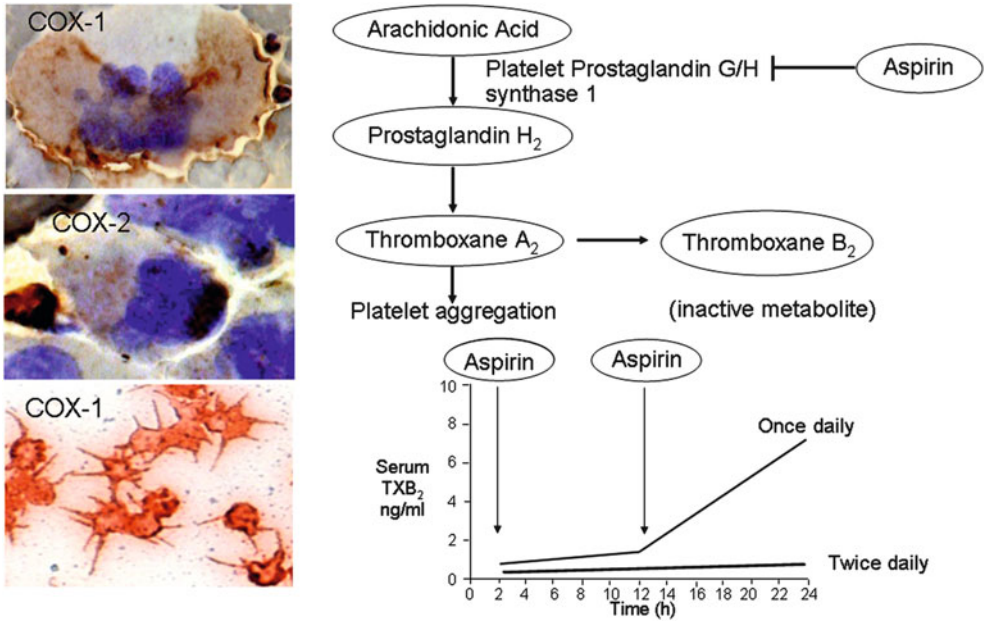

Fig. 3 Model of altered aspirin pharmacodynamics in type 2 diabetes mellitus. Under conditions of normal megakaryopoiesis, low-dose aspirin acetylates COX isozymes in both circulating platelets and bone marrow megakaryocytes, and only negligible amounts of unacetylated enzymes are resynthesized within the 24-h dosing interval. This pharmacodynamic pattern is associated with virtually complete suppression of platelet TXA_2 production in peripheral blood, as reflected by serum TXB_2, throughout the dosing interval. Under conditions of abnormal megakaryopoiesis, an accelerated rate of COX-isozyme resynthesis is biologically plausible in bone marrow megakaryocytes, accompanied by faster release of immature platelets with unacetylated enzyme(s) during the aspirin dosing interval, and in particular between 12 and 24 h after dosing. This pharmacodynamic pattern is associated with incomplete suppression of platelet TXA_2 production in peripheral blood and time-dependent recovery of TXA_2-dependent platelet function during the 24-h dosing interval. Twice daily administration of low-dose aspirin can reverse this abnormal biochemical phenotype. Immunohistochemistry panels depict megakaryocytes stained for COX-1 and COX-2, and peripheral washed platelets stained for COX-1

Collaboration 2013). Meta-analyses of the coxib trials have also provided evidence that some traditional COX-2 inhibitors (e.g., diclofenac and ibuprofen), which only incompletely and transiently inhibit platelet COX-1 activity because of their reversible mechanism of action and short half-life, may increase the risk of major vascular events to a similar extent as the coxibs (Kearney et al. 2006; CNT Collaboration 2013), thereby reinforcing the importance of virtually complete and persistent blockade of platelet COX-1 activity throughout the dosing interval in order to exert cardioprotective effects. In fact, the relationship between inhibition of serum TXB_2 and suppression of TXA_2-dependent platelet activation in vivo is strikingly nonlinear (Reilly and FitzGerald 1987; Santilli et al. 2009), with >97 % inhibition of the former being required for full suppression of the latter (Santilli et al. 2009) (Fig. 4).

Drug–Drug Interactions

Aspirin has been reported to interfere with the antihypertensive effect of blood pressure-lowering drugs, particularly angiotensin-converting enzyme (ACE) inhibitors (Patrono et al. 2008).

This represents a mechanism-based pharmacodynamic interaction that is shared by all NSAIDs and is largely attributed to COX-2 inhibition in the renal cortex and medulla, resulting in increased vascular resistance and sodium retention, respectively (Patrono et al. 2008). This pro-hypertensive effect was not apparent with once daily administration of low-dose (75 mg) aspirin in intensively treated hypertensive patients, in whom this antiplatelet regimen did not impair blood pressure control or the need for antihypertensive therapy as compared to placebo (Hansson et al. 1998).

A subgroup analysis of the PLATO trial, a randomized comparison of ticagrelor vs. clopidogrel in aspirin-treated patients with acute coronary syndromes (ACS) (Wallentin et al. 2009), revealed a potential interaction between aspirin and ticagrelor (Mahaffey et al. 2011). Thus, the higher the daily dose of aspirin (allowed range, 75–325 mg), the lower the benefit of ticagrelor vs. clopidogrel. Although the mechanism of this apparent interaction remains elusive, the FDA has approved ticagrelor for aspirin-treated ACS patients with a warning to maintain the daily aspirin dose at/or lower than 100 mg.

Some NSAIDs, i.e., ibuprofen (Catella-Lawson et al. 2001; Renda et al. 2006) and naproxen (Capone et al. 2005; Li et al. 2014), interfere with the antiplatelet effect

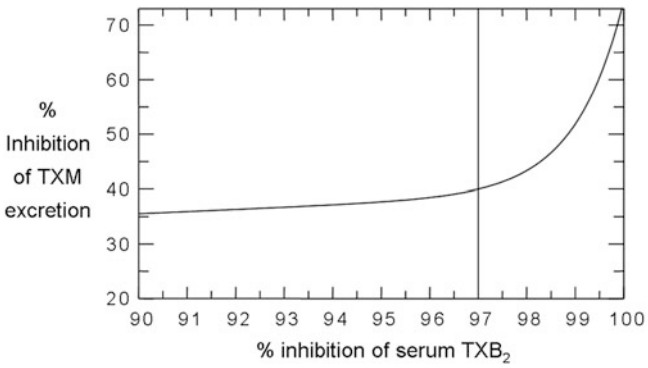

Fig. 4 Nonlinear relationship between inhibition of platelet COX-1 activity, as reflected by serum TXB_2, and inhibition of TXA_2 biosynthesis in vivo, as reflected by urinary 11-dehydro-TXB_2 excretion. Reproduced from Santilli et al. (2009) with permission from the American College of Cardiology

of low-dose aspirin by competing for a common docking site (Arginine-120) within the COX-channel of PGHS-1, to which aspirin binds reversibly with low affinity prior to acetylating Ser-529. Analgesic drugs which have been reported not to share this pharmacodynamic interaction with low-dose aspirin include paracetamol (Catella-Lawson et al. 2001), diclofenac (Catella-Lawson et al. 2001), and celecoxib (Renda et al. 2006), i.e., COX-2 inhibitors with some degree of COX-2 selectivity (FitzGerald and Patrono 2001). The use of these NSAIDs in high-risk patients treated with low-dose aspirin should be restricted to the lowest effective dose for the shortest duration necessary for arthritic symptom control, or else aspirin should be replaced with clopidogrel if the patient is not on dual antiplatelet therapy (Patrono and Baigent 2014).

Clinical Efficacy and Safety

The efficacy and safety of aspirin have been evaluated in several populations, ranging from patients presenting with an acute MI or an acute ischemic stroke to apparently healthy persons at low risk of CV events (Patrono et al. 2005). The highly variable benefit/risk profile of the same antiplatelet strategy in different clinical settings reflects the variable incidence of ischemic vs. hemorrhagic complications in the treated population, as well as the relative importance of TXA_2-dependent platelet activation in coronary vs. cerebrovascular atherothrombosis and in primary hemostasis of the GI tract vs. the brain (Davì and Patrono 2007).

In patients with acute MI, low-dose aspirin (162.5 mg daily) started within 24 h after the onset of symptoms reduced vascular mortality (primary endpoint) by 23 %, as well as recurrent MI or stroke by 50 % (ISIS-2 Collaborative Group 1988). Treatment of 1000 such patients for about 5 weeks resulted in 40 fewer major vascular events with no statistically significant increase in major bleeding complications. In patients with acute ischemic stroke, aspirin (160–300 mg daily) started within 48 h after the onset of symptoms reduced overall mortality by approximately 10 % and improved functional outcome at 30 days (Lansberg et al. 2012). Treatment of 1000 such patients for about 3 weeks resulted in 9 fewer deaths and 7 more patients with a good functional outcome at the expense of 4 more major (nonfatal) extracranial bleeds (Lansberg et al. 2012). Despite the development of newer antiplatelet agents, low-dose aspirin is still recommended with the highest grade and level of evidence (1A) as initial treatment and long-term therapy of patients with ACS (Roffi et al. 2016) or acute ischemic stroke (Lansberg et al. 2012).

In stable patients with atherothrombotic vascular disease, both individual studies (Patrono et al. 2008) and meta-analyses of randomized trials of antiplatelet therapy (ATT Collaboration 2002) indicate that low-dose aspirin reduces the risk of recurrence of a serious vascular event by approximately 25 %. Indirect comparisons of the results of these high-risk trials provide no evidence of a dose-dependent effect within a wide range of aspirin daily doses (30–1500 mg) (ATT Collaboration 2002). Moreover, a limited number of direct, randomized comparisons of higher vs. lower doses of aspirin in patients with cerebrovascular disease or ACS showed no evidence of superiority of higher vs. lower doses, consistent with saturability of the antiplatelet effect at low doses (Patrono 2015) (Fig. 5).

Among a wide range of high-risk patients, in whom the annual rate of a serious vascular event ranges between 4 and 6 %, low-dose aspirin may prevent approximately 10–15 fatal and nonfatal ischemic events for every 1000 patients treated for 1 year (number needed to treat [NNT]: 67–100) (Patrono et al. 2005). This substantial benefit is obtained at the expense of causing 1–2 major extracranial (mostly GI) bleeding complications per 1000 patients (number needed to harm [NNH]: 500–1000) and 1–2 hemorrhagic strokes per 10,000 patients (NNH: 5000–10,000) (Patrono et al. 2005). Therefore, for most patients with a prior vascular event (at average bleeding risk) taking low-dose aspirin, the number in whom the recurrence of a serious vascular event would be avoided clearly outweighs the number in whom aspirin would cause a major bleeding complication (Patrono et al. 2005; Patrono 2015).

In contrast, among asymptomatic subjects without a prior vascular event, the balance of benefits and risks of long-term therapy with low-dose aspirin is substantially uncertain because the risks without aspirin, and hence the absolute benefits of antiplatelet prophylaxis, are about an order of magnitude lower than in secondary prevention (Patrono 2013). Moreover, in contrast to secondary prevention trials, in which a clear benefit of antiplatelet prophylaxis was demonstrated for both fatal and nonfatal coronary and cerebrovascular events, the benefit of aspirin in primary

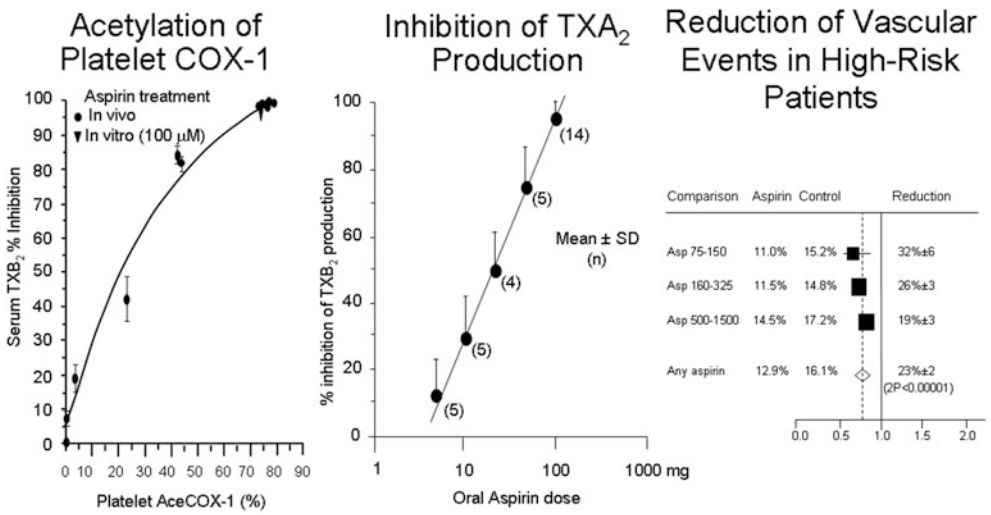

Fig. 5 Acetylation of platelet PGHS-1, inhibition of TXA_2 production, and reduction of vascular events by aspirin. The *left* panel illustrates the saturability of the acetylation process measured in vitro and ex vivo following oral dosing with 100 mg daily in healthy subjects (Patrignani et al. 2014); the *middle* panel depicts saturability of the inhibitory effect on platelet COX-1 activity, as reflected by serum TXB_2 measurements after single oral doses of aspirin in healthy subjects (Patrignani et al. 1982); the *right* panel illustrates saturability of the clinical effect of aspirin in reducing risk of serious vascular events in high-risk patients (ATT Collaboration 2002)

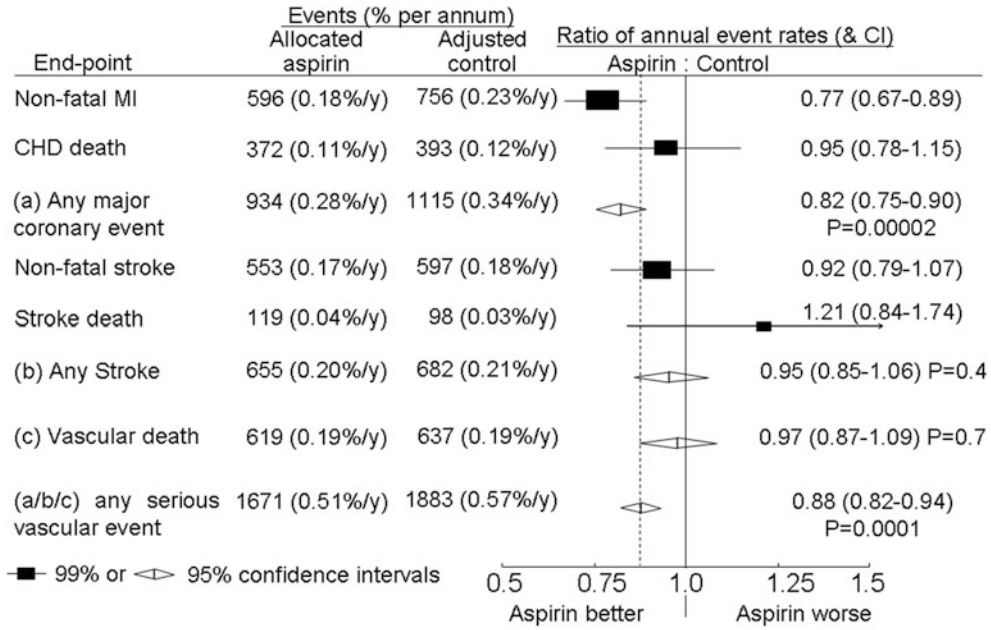

Fig. 6 Serious vascular events in primary prevention trials and proportional effects of aspirin allocation. Actual numbers for aspirin-allocated trial participants, and adjusted numbers for control-allocated trial participants, are presented, together with the corresponding mean yearly event rate (in *parentheses*). Rate ratios (RRs) for all trials are indicated by *squares* and their 99 % CIs by *horizontal lines*. Subtotals and their 95 % CIs are represented by *diamonds*. Squares or diamonds to the *left* of the *solid line* indicate benefit. *MI* myocardial infarction, *CHD* coronary heart disease. (asterisk) Myocardial infarction, stroke, or vascular death. Vascular death is coronary heart disease death, stroke death, or other vascular death (which includes sudden death, death from pulmonary embolism, and death from any hemorrhage but in the primary prevention trials excludes death from an unknown cause). Reproduced from ATT Collaboration (2009) with permission from The Lancet

prevention trials was limited to reducing the risk of nonfatal MI (ATT Collaboration 2009) (Fig. 6). On the basis of a meta-analysis of individual participant data from the six largest primary prevention trials of aspirin in approximately 90,000 subjects at relatively low vascular risk (0.6 % per year), the ATT Collaboration (2009) has shown that, irrespective of age or sex, the absolute reduction in serious vascular events would be only about twice as large as the

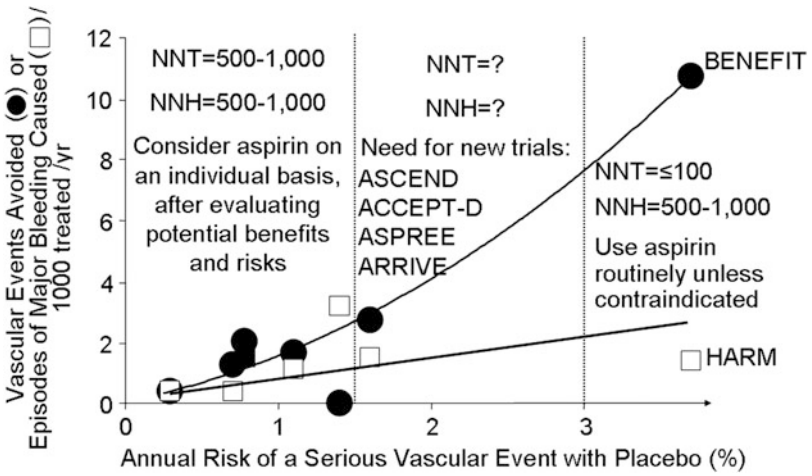

Fig. 7 The numbers of vascular events avoided and episodes of major bleeding caused per 1000 patients treated with aspirin per year are plotted from the results of individual placebo-controlled trials of aspirin in different patient populations characterized by various degrees of cardiovascular risk, as noted on the abscissa. Number needed to treat (NNT) and number needed to harm (NNH) values are given for subjects in three categories of risk on the basis of the presence or absence of randomized controlled trials. *ACCEPT-D* Aspirin and Simvastatin Combination for Cardiovascular Events Prevention Trials in Diabetes, *ASCEND* A Study of Cardiovascular Events in Diabetes, *ARRIVE* Aspirin to Reduce Risk of Initial Vascular Events, *ASPREE* ASPirin in Reducing Events in the Elderly, *BDT* British Doctors Trial, *HOT* Hypertension Optimal Treatment Study, *PHS* Physicians' Health Study, *PPP* Primary Prevention Project, *SAPAT* Swedish Angina Pectoris Aspirin Trial, *TPT* Thrombosis Prevention Trial, *WHS* Women's Health Study. Reproduced from Patrono (2015) with permission from the American College of Cardiology

absolute increase in nonfatal GI bleeding. Moreover, the predicted 5-year absolute effects of allocation to aspirin in subjects at low to high coronary risk (5-year risk from <5 % to >10 %) would yield a relatively constant ratio between the calculated NNH (from 1000 to 100, respectively) and NNT values (from 500 to 50, respectively) (ATT Collaboration 2009). This is not surprising, as the main risk factors for coronary events (with the exception of hypercholesterolemia) were also associated with bleeding complications (ATT Collaboration 2009). The current uncertainty on the benefit/risk profile is reflected by conflicting guidelines on the use of aspirin in primary prevention, as well as by its heterogeneous regulatory status in different countries (Patrono 2015).

Based on the available results of primary and secondary prevention trials, one can identify three areas of CV risk (Fig. 7): (1) one with an annual risk up to 1.5 %, where evidence from five randomized trials allows a reliable calculation of both NNT and NNH values to guide a personalized approach to antiplatelet prophylaxis; (2) an intermediate zone between 1.5 and 3 % annual CV risk, where evidence from a single trial is insufficient to reliably assess benefit and harm and new trials are needed (see below) to address this knowledge gap; and (3) an area of annual risk >3 %, starting with patients with chronic stable angina, where the evidence from many trials shows that the benefit of aspirin treatment clearly outweighs any potential harm resulting from bleeding complications (Patrono 2015).

Several lines of evidence support a chemopreventive effect of aspirin against cancer of the GI tract, particularly colorectal cancer (Thun et al. 2012), through a mechanism of action possibly related to platelet inhibition (Patrignani and Patrono 2016) (Fig. 8). It has been argued that even a 10 % reduction by low-dose aspirin in overall cancer incidence over 5 years would yield an absolute benefit comparable to the absolute reduction in serious vascular events; these combined benefits would outweigh the absolute excess of major bleeding complications by three- to fivefold, depending on age and gender (Thun et al. 2012). The US Preventive Services Task Force (Bibbins-Domingo et al 2016) has recently published a recommendation statement addressing the possibility that the same antiplatelet regimen of low-dose aspirin may be recommended for the primary prevention of both CV disease and colorectal cancer in adults ages 50–59 years who have a 10 % or greater 10-year CVD risk, are not at increased risk for bleeding, and have a life expectancy of at least 10 years.

Low-dose aspirin has been reported to produce additional benefits in the prevention of venous thromboembolism (Patrono 2015) and preeclampsia (Mol et al. 2015) that may well reflect the participation of TXA_2-dependent platelet activation in their pathophysiology (Fig. 8).

Ongoing Trials

Several ongoing, placebo-controlled trials may help assess the benefit/risk profile of low-dose aspirin in preventing CV complications and other outcomes (including dementia and cancer) in approximately 50,000 participants at higher CV

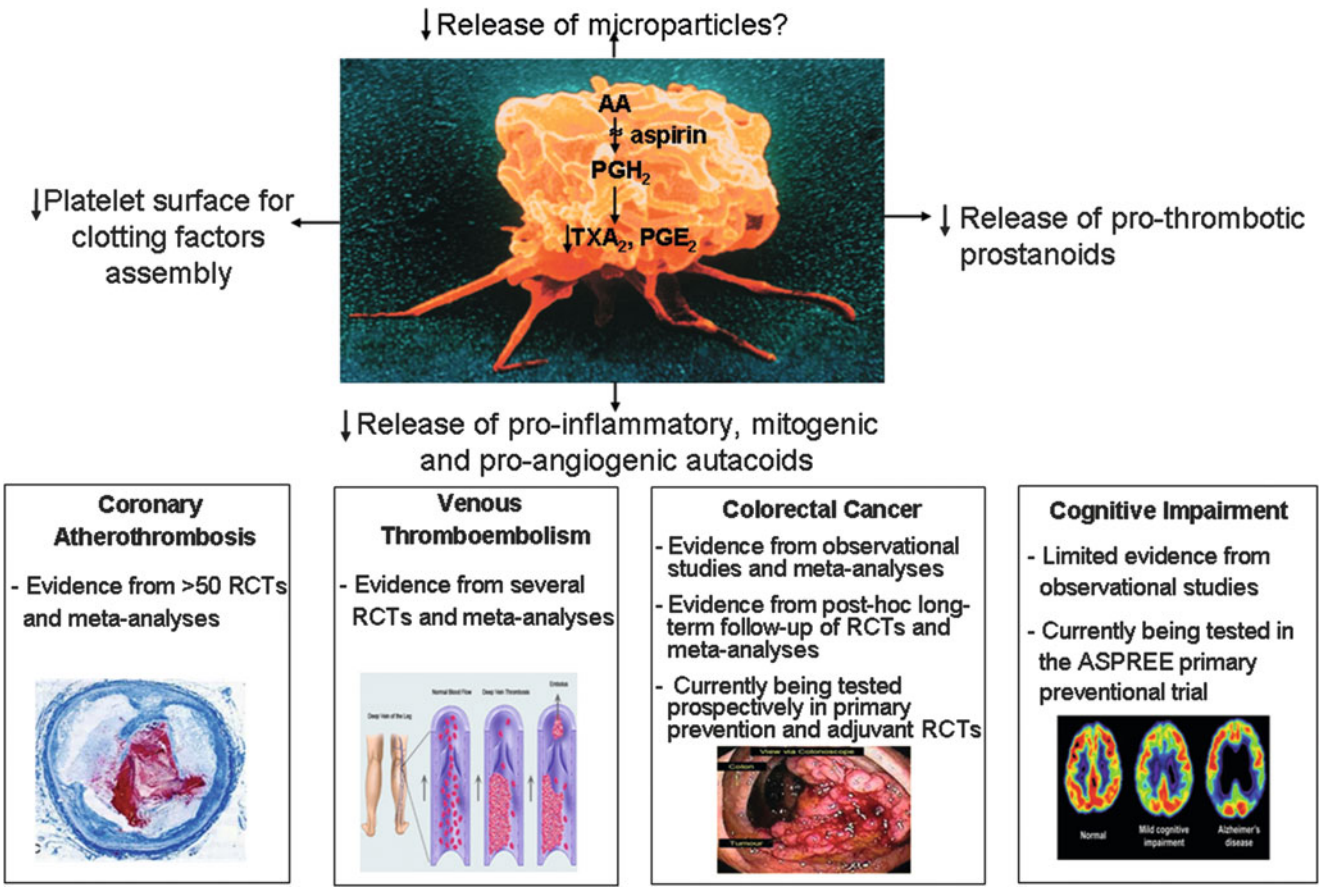

Fig. 8 A wide repertoire of lipid and protein mediators, which may contribute to several clinical syndromes possibly responsive to low-dose aspirin therapy, are released upon platelet activation and aggregation. The figure illustrates inhibition of platelet prostanoid production by aspirin and its functional and clinical consequences. The lines of evidence supporting the protective effects of aspirin are summarized below each panel. *AA* arachidonic acid, *ASPREE* ASPirin in Reducing Events in the Elderly, *RCT* randomized controlled trial(s), PGE_2 prostaglandin E_2, PGH_2 prostaglandin H_2, TXA_2 thromboxane A_2. Reproduced from Patrono (2015) with permission from the American College of Cardiology

risk than in the earlier trials because of diabetes mellitus (ASCEND [A Study of Cardiovascular Events in Diabetes], British Heart Foundation 2015; and ACCEPT-D [Aspirin and Simvastatin Combination for Cardiovascular Events Prevention Trials in Diabetes], De Berardis et al. 2007), advanced age (ASPREE [ASPirin in Reducing Events in the Elderly], ASPREE Investigator Group 2013), or a cluster of CV risk factors (not including diabetes) expected to confer a 10-year risk >15 % (ARRIVE [Aspirin to Reduce Risk of Initial Vascular Events], Bayer HealthCare 2015). The inclusion of colorectal cancer as a prespecified secondary endpoint in these trials will allow collecting a large amount of prospective data on the chemopreventive effect of low-dose aspirin during a 5- to 7.5-year follow-up (Patrono 2015). In addition, a number of adjuvant trials in cancer patients (e.g., ADD-Aspirin trial) are currently ongoing or planned in which participants undergone primary treatment with curative intent for an early-stage common solid tumor (e.g., colorectal, gastroesophageal, breast, or prostate) are randomized to receive aspirin (one or two daily doses in the range 100–300 mg) or placebo for 1–8 years and will be assessed for disease-free survival or overall survival (Patrono 2015) (Fig. 9).

Gaps in Knowledge and Future Directions for Research

The last 35 years of clinical research on aspirin have proven its value as a lifesaving drug for the treatment and prevention of atherothrombosis (Patrono et al. 2005). The place of low-dose aspirin in the therapeutic armamentarium has not been substantially altered by the successful development of at least ten new antiplatelet agents, including $P2Y_{12}$ and PAR-1 receptor blockers (Patrono and Rocca 2010). The demonstration of additive beneficial effects resulting from effective blockade of the platelet ADP and/or thrombin receptor on top of TXA_2 suppression in high-risk patients

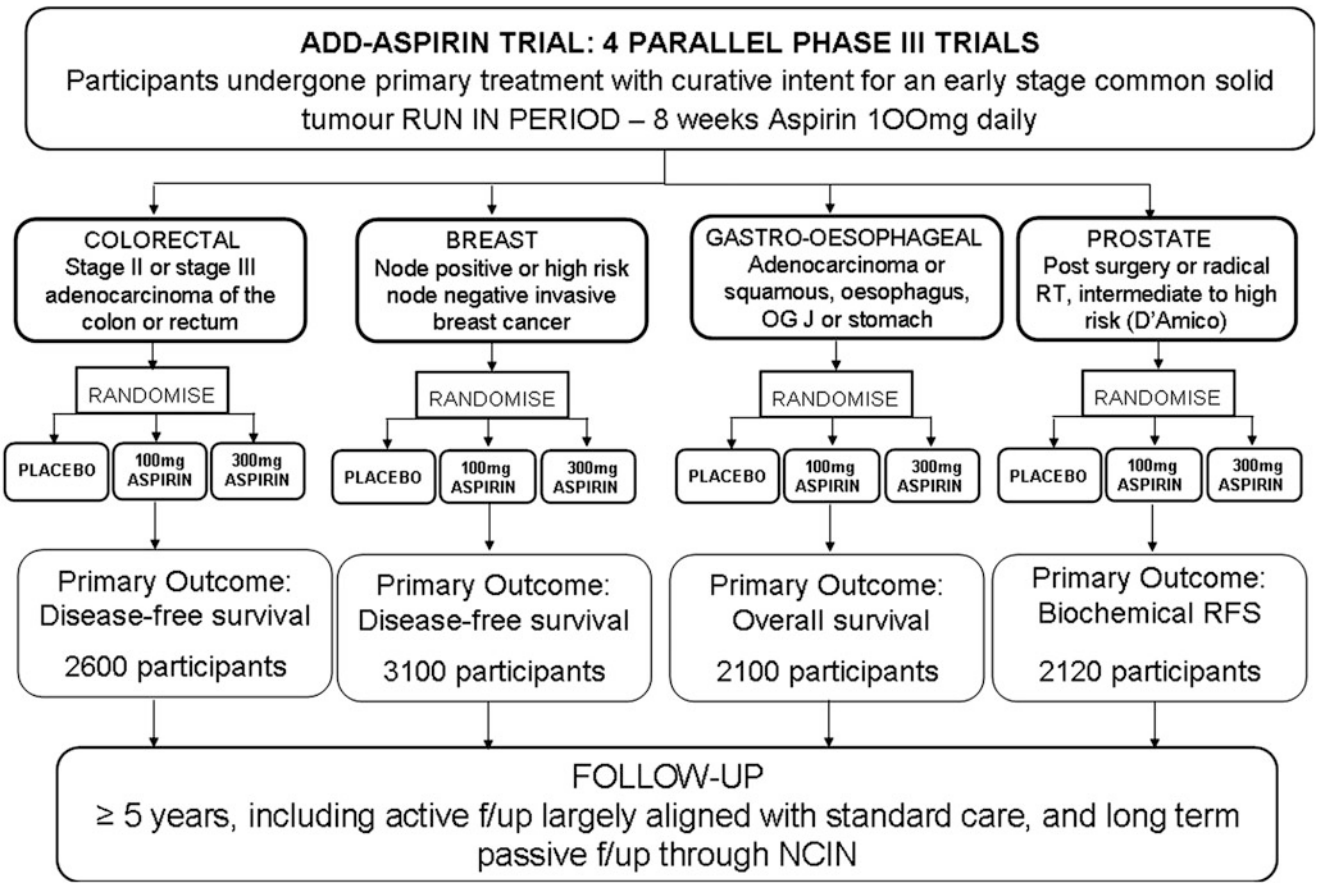

Fig. 9 Design of the ADD-Aspirin trial, four parallel phase-3 trials in patients with colorectal, breast, gastroesophageal, or prostate cancer. Courtesy of Prof. Ruth Langley, University College London, UK

is consistent with the multifactorial nature of atherothrombosis and the nonredundant nature of these different pathways of platelet activation (Davì and Patrono 2007). However, the size of the residual CV risk in patients with ACS (about 10 % experiencing a major vascular event at 1 year) despite optimal pharmacological treatment, including low-dose aspirin, effective $P2Y_{12}$ inhibitors and statins, calls for reappraisal of the pathophysiology of these adverse outcomes and innovative preventive strategies (Roffi et al. 2016). Furthermore, several randomized trials are underway to determine whether aspirin can be dropped from combined antiplatelet regimens for the long-term management of patients who are treated with one of the new antiplatelet drugs (Vranckx et al. 2015).

Besides becoming an essential component of the antithrombotic strategy in high-risk settings, low-dose aspirin has also provided a mechanistic insight into the participation of platelets in other pathophysiologic processes. The hypothesis that platelet activation during the repair process of intestinal mucosal lesions may trigger an early event in colorectal carcinogenesis, i.e., COX-2 induction in adjacent nucleated cells and increased PGE_2 production, was formulated on the basis of the lack of a dose effect in the apparent protection against cancer development and death associated with aspirin use in observational studies (Patrono et al. 2001), a finding supported by post hoc analyses of CV prevention trials of aspirin showing saturability of the apparent chemopreventive effect at low doses (Thun et al. 2012). This hypothesis is being tested prospectively in the ongoing primary prevention and adjuvant trials mentioned above (Patrono 2015).

Similarly, the potential role of platelet-derived inflammatory mediators in neurodegeneration and cognitive decline is currently being explored by the ASPREE Investigator Group (2013) in a placebo-controlled aspirin trial of 19,000 elderly subjects with no diabetes or CVD who are being followed for 5 years with death, dementia, or significant disability as the primary endpoint. Measurement of noninvasive biomarkers of platelet activation in substudies of the ongoing trials may allow further characterization of the role of

platelet activation and inhibition in atherothrombotic and other important disorders sharing common mechanisms of disease.

Take-Home Messages

- Low-dose aspirin remains the cornerstone of antiplatelet therapy in the treatment of acute ischemic syndromes and in the secondary prevention of atherothrombosis.
- The place of aspirin in primary prevention remains controversial because of the uncertain balance of benefits vs risks, requiring a personalized approach to therapy that takes into consideration current knowledge as well as patient's values and preferences.
- Additional research is needed to characterize the mechanism of action and optimal dose of aspirin in the chemoprevention of colorectal cancer, a potential additional benefit of long-term antiplatelet therapy.

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Pharmacology: Inhibitors of $P2Y_{12}$

Lisa Gross, Dániel Aradi, and Dirk Sibbing

Abstract

Dual antiplatelet treatment consisting of aspirin and a $P2Y_{12}$ inhibitor represents the cornerstone of treatment in patients with acute coronary syndrome (ACS) and those undergoing percutaneous coronary intervention. $P2Y_{12}$ inhibitors are broadly used worldwide to prevent and treat ischemic events in cardiac patients; however, understanding their pharmacokinetic and pharmacodynamic properties is important for accurate use in daily practice.

Within the group of $P2Y_{12}$ inhibitors, thienopyridines such as ticlopidine, clopidogrel, and prasugrel are prodrugs that require hepatic metabolization and conversion to a pharmacologically active metabolite to exert the antiplatelet effect. Their active metabolites covalently and irreversibly bind to the $P2Y_{12}$ receptor for the entire life span of the platelet. Contrary, the cyclopentyltriazolopyrimidine-type $P2Y_{12}$ inhibitor, ticagrelor, and the intravenous $P2Y_{12}$ receptor inhibitor cangrelor are direct-acting $P2Y_{12}$ inhibitors that do not require metabolic activation and noncompetitively inhibit the $P2Y_{12}$ receptor by inducing a conformational change.

Due to lower risk of hematological side effects, faster onset of action, and once-daily administration, ticlopidine was soon replaced by clopidogrel that has become the $P2Y_{12}$ inhibitor of choice for many years in patients with cardiovascular disease. However, the onset of the antiplatelet effect of clopidogrel was still relatively delayed for many ACS patients requiring urgent PCI. In addition, a relatively high rate (15–40 %) of patients did not reach adequately low levels of platelet reactivity with clopidogrel—as evaluated by ADP-induced platelet function testing—and was exposed to an increased risk of stent thrombosis, myocardial infarction, and mortality. Thus, novel oral $P2Y_{12}$ receptor inhibitors prasugrel and ticagrelor were developed to provide faster, more predictable,

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and more potent antiplatelet effect than clopidogrel. Approved by the FDA in 2009 and 2011, respectively, both prasugrel and ticagrelor showed improved thrombo-ischemic outcomes in patients with ACS, albeit at an expense of increased risk for bleeding. Despite being a reversibly binding agent, 5 days is required for the recovery of normal platelet function after discontinuation of ticagrelor. With this regard, cangrelor, the first intravenous $P2Y_{12}$ inhibitor, has a unique pharmacokinetic profile with a 5–10-min plasma half-life resulting in immediate, potent $P2Y_{12}$ inhibition with a complete offset of action within 30–60 min after discontinuation.

This book chapter aims to provide further insights regarding the pharmacokinetic and pharmacodynamic properties of available $P2Y_{12}$ inhibitors assisting their everyday use in clinical practice.

Abbreviations

ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
COX-1	Cyclooxygenase 1
FDA	US Food and Drug Administration
PCI	Percutaneous coronary intervention

Introduction

Platelet activation and aggregation are fundamental steps in the development of acute coronary events, and platelets also play a key role in the progression of atherosclerosis (Davi and Patrono 2007). Therefore, antiplatelet agents represent some of the most commonly prescribed drugs worldwide to prevent and treat cardiovascular events.

Platelet adhesion and subsequent activation may occur at sites of vascular injury, rupture of atherosclerotic plaques, or in the vicinity of exposed struts of implanted coronary stents. The activation process is triggered and amplified by three major agonists: thrombin, adenosine diphosphate (ADP), and thromboxane A_2 leading to the formation of platelet-rich thrombus (see Fig. 1) (Wallentin 2009).

ADP, released from the dense granules of all activated platelets, is a major driver of thrombus formation. ADP stimulates platelet activation through two G-protein-coupled receptors, $P2Y_1$ and $P2Y_{12}$. $P2Y_1$ is a Gq-coupled receptor that initiates ADP-induced platelet aggregation through the stimulation of phospholipase C and phosphatidylinositol-signaling pathway (Kalantzi et al. 2012). $P2Y_1$ receptor activation initiates shape change and early, slight, and reversible aggregation, without stable thrombus formation. Binding of ADP to the $P2Y_{12}$ receptor results in signaling cascades that amplify and sustain platelet aggregation, as well as strongly increase platelet degranulation and procoagulant activity, which culminates in thrombus growth and stabilization (Wallentin 2009; Daniel et al. 1999; Davi and Patrono 2007; Dorsam and Kunapuli 2004). $P2Y_{12}$ is a Gi-coupled seven-transmembrane domain receptor (see Fig. 2), which mediates platelet activation by inhibiting the adenylate cyclase-mediated signaling pathway and decreasing intracellular cAMP levels. The intercellular signaling pathways triggered by $P2Y_1$ receptor and $P2Y_{12}$ receptor activation and their physiological effects are illustrated in Fig. 5. The overall fold of the P2Y12R structure consists of a canonical seven-transmembrane (7TM) bundle of α-helices and a carboxy-terminal helix VIII that is parallel to the membrane bilayer (Zhang et al. 2014). The $P2Y_{12}$ receptor couples primarily to $G\alpha_{i2}$ and less prominently to other members of the Gi family, resulting in the inhibition of adenylyl cyclase. Rap1b, Akt, and potassium channels are important functional effectors downstream of $P2Y_{12}$ receptor stimulation (Wallentin 2009; Kalantzi et al. 2012; Kim et al. 2004). The decrease in intracellular cAMP levels reduces the rate of phosphorylation of the vasodilator-stimulated phosphoprotein, thus inducing activation of the $\alpha_{IIb}\beta_3$ receptor and platelet aggregation. Thus, $P2Y_{12}$ plays a central role in amplification and stabilization of ADP-induced platelet aggregation (Kalantzi et al. 2012).

Thienopyridines, such as ticlopidine, clopidogrel, and prasugrel, are inactive prodrugs and need to undergo hepatic metabolism to generate an active metabolite that binds covalently and irreversibly to the ADP-binding site on the $P2Y_{12}$ receptor for the entire life span of the platelet (Wallentin 2009; Michelson 2008). Contrary, ticagrelor and cangrelor do not belong to the thienopyridine class, but are direct-acting $P2Y_{12}$ inhibitors that reversibly change the conformation of the $P2Y_{12}$ receptor (see Fig. 4).

Differences in absorption, metabolism, receptor binding, and drug interactions all influence the final $P2Y_{12}$ inhibitory effect of the specific agent. This book chapter aims to provide insights into the pharmacokinetic and pharmacodynamic properties of $P2Y_{12}$ inhibitors.

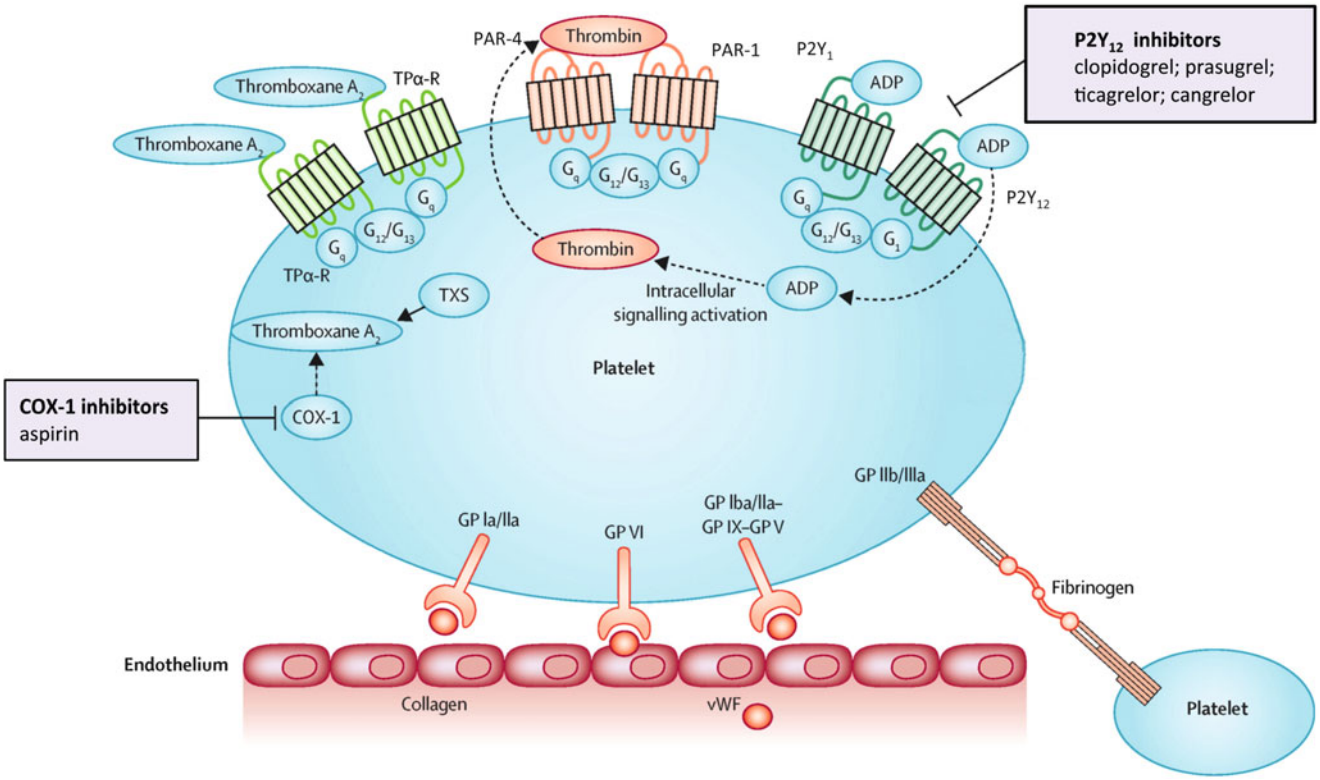

Fig. 1 Platelet pathways targeted by $P2Y_{12}$ inhibitors and aspirin. Platelet adherence to injured vascular endothelium is mediated by binding of glycoprotein receptors to exposed extracellular matrix proteins (collagen and vWF). Complex intracellular signaling processes result in the local production and release of multiple agonists, including thromboxane A2 and ADP. These factors bind to their respective G-protein-coupled receptors, mediating paracrine and autocrine platelet activation. Platelet integrin $\alpha_{IIb}\beta_3$ mediates the final common step of platelet aggregation by undergoing a conformational change, binding fibrinogen and vWF, and leading to platelet-rich thrombus. Dual antiplatelet therapy consisting of aspirin and a $P2Y_{12}$ inhibitor effectively targets pathways associated with thromboxane A2 and ADP-mediated platelet activation. *vWF* von Willebrand factor, *GP* glycoprotein, *COX-1* cyclooxygenase *1, 5-HT2A* serotonin receptor 2A, *PAR* protease-activated receptor, *TP* thromboxane prostanoid receptor, *TXS* thromboxane A synthase. Modified from Franchi and Angiolillo (2015), by permission of Nature Reviews Cardiology

Ticlopidine

Ticlopidine (see Fig. 3) was the first thienopyridine and received US Food and Drug Administration (FDA) approval in 1991 for the prevention of ischemic events in patients with coronary artery disease.

Ticlopidine is administered orally in a dose of 250 mg twice daily. Onset of action occurs between 24 and 48 h after initiation of treatment, with maximal inhibition of ADP-induced platelet aggregation 3–5 days after initial dosing (see Table 1) (Farid et al. 2010; Picard-Fraire 1983; Saltiel and Ward 1987). Eighty-five percent of the drug is absorbed, with peak plasma concentrations (C_{max}) being reached 1–3 h after the oral dose (Farid et al. 2010; Picard-Fraire 1983). The median ticlopidine C_{max} after the first oral dose is approx. 310 ng/mL, increasing to 990 ng/ml after 21 days of treatment (Farid et al. 2010; Knudsen et al. 1992). Pharmacokinetics is nonlinear, with significant decrease in clearance after repeated dosing. The median half-life ($t_{1/2}$) after multiple dosing is 29 h, but may be up to 4–5 days in elderly patients (Farid et al. 2010; Knudsen et al. 1992)—Likewise, steady-state concentrations in plasma are achieved in 14–21 days in elderly patients, but within 5 days in younger patients (Farid et al. 2010; Saltiel and Ward 1987). Ticlopidine is 98 % plasma protein-bound (Ito et al. 1992). It is a prodrug, requiring multistep hepatic CYP P450-dependent bioactivation to form the active metabolite, which was isolated and characterized by Yoneda et al. in 2004 (see Fig. 4) (Yoneda et al. 2004). It shows a rapid hepatic metabolism and is metabolized by at least five main pathways resulting in a minimum of 13, mostly inactive, metabolites (Wallentin 2009; Picard-Fraire 1983). CYP2C19, CYP2B6, and CYP3A pathways have been shown to contribute to the metabolism of ticlopidine (Ha-Duong et al. 2001; Richter et al. 2004; Dalvie and O'Connell 2004). However, the CYPs involved in the formation of the active metabolite from its thiolactone

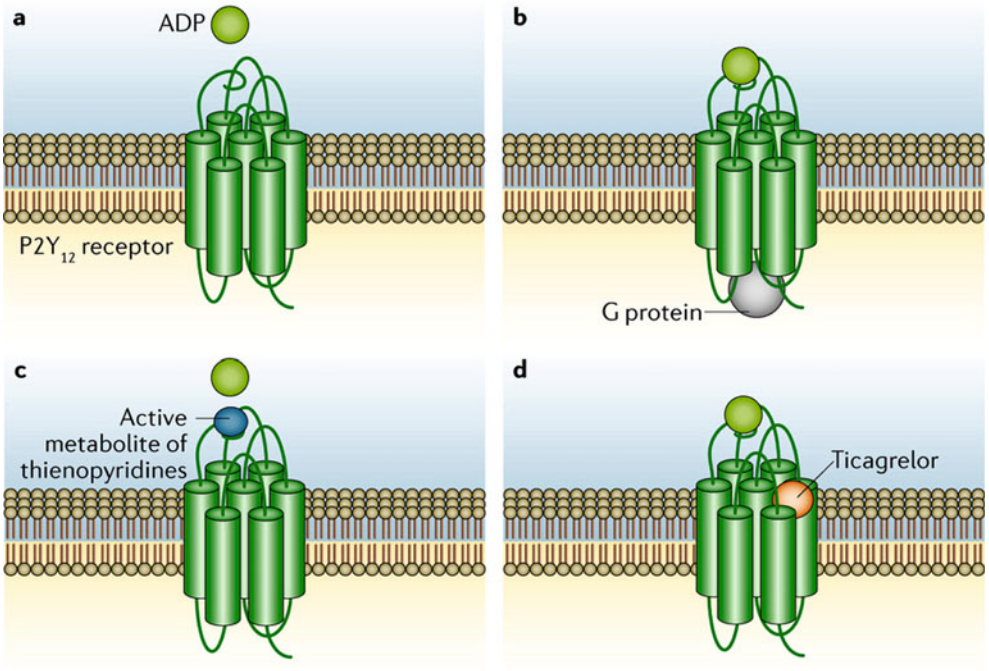

Fig. 2 Binding properties of $P2Y_{12}$ receptor inhibitors. (**a**) ADP binds to the P2Y12 receptor, which (**b**) leads to a conformational change of the receptor and to G-protein activation. (**c**) Competitive binding of the active metabolite of thienopyridines to the $P2Y_{12}$ receptor. Binding is irreversible, which renders the receptor nonfunctional for the life of the platelet. (**d**) Ticagrelor binds reversibly to the $P2Y_{12}$ receptor at a site that is distinct from the ADP-binding site (noncompetitive binding). Ticagrelor inhibits ADP signaling and conformational change of the receptor by "locking" the receptor in an inactive state; the receptor is functional after dissociation of the ticagrelor molecule. ADP can still bind at its binding site, and the degree of receptor inhibition is dependent on the concentration of ticagrelor. Reprinted with permission from Fabiana Rollini, Francesco Franchi, and Dominick J. Angiolillo, Nature Reviews Cardiology 2016 (Rollini et al. 2016), by permission of Nature Reviews Cardiology

intermediate are still unknown (Farid et al. 2010). 60 % of the agent is eliminated in the urine and 23 % in the feces.

The most common side effects observed with ticlopidine included diarrhea, nausea, vomiting, stomach pain, purpura, and skin rashes (Whetsel and Bell 1999). Patients treated with ticlopidone also showed potentially life-threatening side effects including hepatotoxicity (Martinez Perez-Balsa et al. 1998; Takikawa 2005), hyponatremia, nephrotic syndrome, neutropenia, and thrombocytopenia (Steinhubl et al. 1999). Due to these toxic side effects and its slow onset of action, ticlopidine was replaced by clopidogrel (Berger et al. 1999; Muller et al. 2000; Quinn and Fitzgerald 1999; Moussa et al. 1999).

Clopidogrel

Clopidogrel (see Fig. 3) is a second-generation thienopyridine and was approved by the FDA in 1997 to reduce the incidence of ischemic events in patients with atherosclerotic vascular disease. It is a prodrug, and its active metabolite covalently and irreversibly binds to the $P2Y_{12}$ receptor for the entire life span of the platelet (see Figs. 2 and 5).

The pharmacokinetic and pharmacodynamic properties of clopidogrel are summarized in the Table 1. The recommended oral dose is 75 mg once daily. The peak plasma concentrations of the parent carboxylic metabolite as well as the active metabolite (see Fig. 4) occur within 30 min to 1 h following administration of a single oral dose (Small et al. 2008). Administration of a loading dose between 300 and 600 mg leads to a peak platelet inhibition response within 2–5 h (Muller et al. 2001; Wallentin 2009; von Beckerath et al. 2005; Price et al. 2006; Montalescot et al. 2006). The C_{max} of clopidogrel active metabolite after a 300-mg loading dose is approximately 70 ng/mL and approximately 30 ng/mL after a 75-mg maintenance dose (Lenz and Wilson 2003). Bonello et al. showed in 2008 that a 600-mg loading dose was associated with a significant decrease in the rate of post-PCI major adverse cardiovascular events at 1 month compared with a 300-mg loading dose, without any inhospital increase in bleeding complications (95 % confidence interval 0.41–0.95, $p = 0.03$) (Bonello et al. 2008b). Furthermore, it has been shown that loading

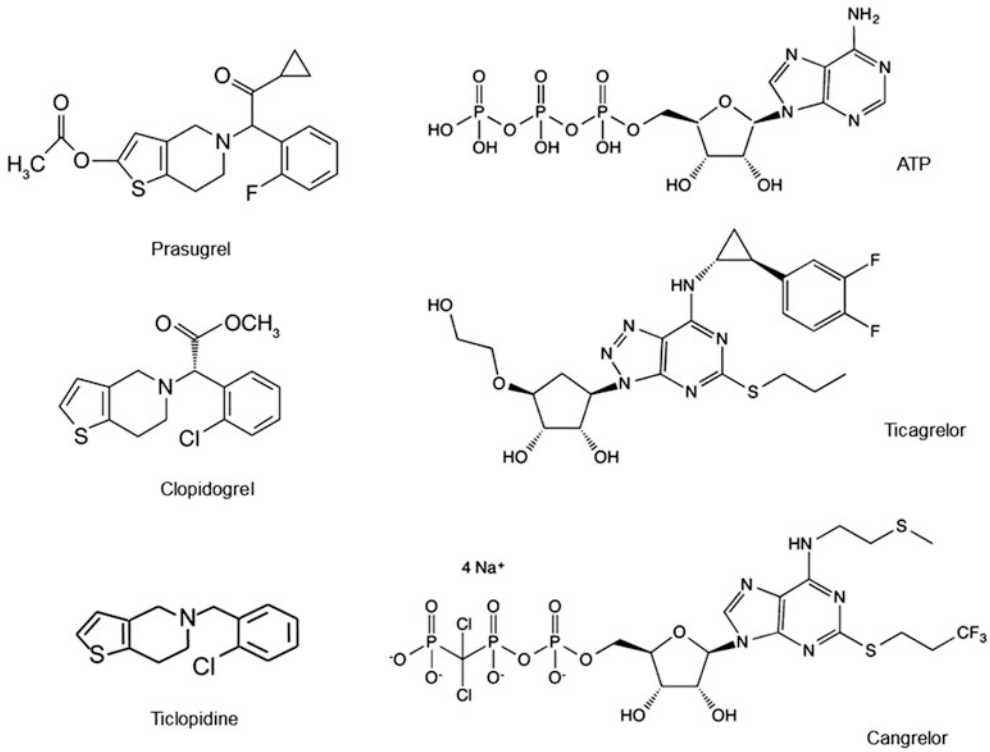

Fig. 3 Chemical structures of prasugrel, clopidogrel, ticlopidine, adenosine triphosphate (ATP), ticagrelor, and cangrelor

doses exceeding 600 mg only slightly, but not significantly, increased platelet inhibitory capacity (von Beckerath et al. 2005). However, in patients who were nonresponders to clopidogrel despite a 600-mg loading dose, adjusting the clopidogrel loading dose according to platelet monitoring using the VASP index has been shown to significantly improve the clinical outcome after PCI (Bonello et al. 2008a, 2009). Gladding et al. showed that in PCI patients a clopidogrel 1200-mg loading dose, given as two 600-mg doses 2 h apart, results in more rapid and complete platelet inhibition than a single 600-mg dose and a maintenance dosage of 150 mg daily produces greater platelet inhibition than 75 mg daily (Gladding et al. 2008). In line, Angiolillo et al. showed that the use of a 150-mg maintenance dose of clopidogrel in patients with type 2 diabetes with <50 % platelet inhibition is associated with enhanced antiplatelet effects. However, the antiplatelet effects achieved were nonuniform, and a considerable number of patients persisted with inadequate platelet inhibition (Angiolillo et al. 2007b, 2008).

The achieved level of platelet inhibition is generally maintained until dosing is discontinued (Jernberg et al. 2006; Wallentin et al. 2008; Savcic et al. 1999; Caplain and Cariou 1999; Thebault et al. 1999). Platelet inhibition decreases to pretreatment levels 5–7 days after treatment is terminated, but the recovery is dependent on the platelet inhibitory effect before discontinuation (Caplain et al. 1999; Caplain and Cariou 1999; Thebault et al. 1999; Weber et al. 2001).

Clopidogrel is rapidly absorbed through adenosine triphosphate binding cassette efflux transporters located in the apical membrane of the intestinal mucosa (Taubert et al. 2006). Both the parent drug and the active metabolite are highly protein bound (~98 % and ~94 %). As a prodrug, clopidogrel requires metabolic transformation to exert its antiplatelet effect (see Table 1) (Angiolillo 2012). Approximately 85 % of clopidogrel is hydrolyzed in the blood by esterases to an inactive carboxyl metabolite. Only about 15 % of the absorbed prodrug is rapidly metabolized by hepatic cytochrome P450 isoenzymes (including CYP 3A4/5, CYP 2C19, CYP 1A2, CYP 2C9, and CYP 2B6) in a two-step oxidation process to a highly unstable active metabolite (see Figs. 4 and 5) (Franchi and Angiolillo 2015). The active metabolite belongs to a family of eight isomers with chemical structure of 2-{1-[1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene} acetic acid (Pereillo et al. 2002). Approximately 50 % of clopidogrel are excreted in the urine and 46 % in the feces in the form of inactive metabolites, respectively (Lins et al. 1999). Owing to the extreme labile nature of the active metabolite, proper characterization of the elimination kinetics of the active metabolite has not been performed (Mullangi and Srinivas 2009).

Clopidogrel is the most widely used $P2Y_{12}$ inhibitor, recommended in patients with stable coronary artery disease undergoing PCI, in patients after stroke, and in those with peripheral artery disease (Franchi and Angiolillo 2015; Sherwood et al. 2014; Fihn et al. 2014; Task Force et al.

Table 1 Pharmacological properties of $P2Y_{12}$ receptor inhibitors

	Ticlopidine	Clopidogrel	Prasugrel	Ticagrelor	Cangrelor
Route of administration	Oral	Oral	Oral	Oral	Intravenous
Dose	250 mg twice daily	LD 300/600 mg, MD 75 mg once daily	LD 60 mg, MD 10 mg once daily (5 mg if <60 kg, >75 years)	LD 180 mg, MD 90 mg twice daily	30 μg/kg bolus, 4 μg/kg/min infusion
Receptor blockade	Irreversible	Irreversible	Irreversible	Reversible	Reversible
Metabolism	Prodrug CYP P450 dependent (multistep)	Prodrug CYP P450 dependent (2 steps)	Prodrug Intestinal esterase + CYP P450 dependent (single hepatic step)	Direct-acting No biotransformation needed, CYP P450-dependent active metabolite	Direct-acting No biotransformation
Onset of action (after LD)	24–48 h	2–5 h	30 min–5 h	30 min–5 h	2 min
Offset of action	11–13 days	5–7 days	7–10 days	5–7 days	30–60 min
Time to peak plasma concentration, of AM t_{max}	1–3 h (Yoneda et al. 2004)	30 min–1 h (Small et al. 2008)	30 min (Small et al. 2008)	2 h (Husted et al. 2012)	2 min (Teng et al. 2010)
IPA $_{MD}$, % (LTA-defined)	20–30 % (Sharis et al. 1998; Kereiakes et al. 1997; Farid et al. 2010) (highly variable)	34–52 % (Farid et al. 2008, 2010) (highly variable)	50–71 % (Farid et al. 2008, 2010)	58–93 % (Gurbel et al. 2009; Husted et al. 2006)	>80 % (Franchi and Angiolillo 2015; Storey et al. 2001)
Phase III clinical trials	STARS (Leon et al. 1998) ISAR (Schomig et al. 1996)	CAPRIE (Committee 1996) CURE (Yusuf et al. 2001) CLARITY-TIMI 28 (Sabatine et al. 2005) COMMIT (Chen et al. 2005) CREDO (Steinhubl et al. 2002) CHARISMA (Bhatt et al. 2004) CURRENT-OASIS 7 (Mehta et al. 2008) COGENT (Bhatt et al. 2010) FASTER (Kennedy et al. 2007) MATCH (Diener et al. 2004) CHANCE (Wang et al. 2013)	TRITON-TIMI 38 (Wiviott et al. 2007a) ACCOAST (Montalescot et al. 2013) TRILOGY-ACS (Roe et al. 2012)	PLATO (Wallentin et al. 2009) PEGASUS	CHAMPION-PCI (Harrington et al. 2009) CHAMPION-PLATFORM (Bhatt et al. 2009) CHAMPION-PHOENIX (Bhatt et al. 2013)

IPA inhibition of platelet aggregation, *MD* maintenance dose, *CAD* coronary artery disease, *ACS* acute coronary syndrome, *PCI* percutaneous coronary intervention, *h* hours, *min* minutes, *CYP P450* cytochromes P450

2013; Levine et al. 2015; Task Force on the management of et al. 2012).

An important limitation of clopidogrel is that the generation of active metabolites and consequently the achieved level of platelet inhibition is highly variable after fixed dosing. This is primarily due to single nucleotide polymorphisms in key genes of the CYP system that catalyze clopidogrel activation: patients having loss-of-function copies (most commonly *2 and *3) of the CYP2C19 gene have lower levels of active metabolites and higher levels of platelet reactivity despite treatment. In turn, those with gain-of-function copies (like *17) of the CYP2C19 have higher exposure to clopidogrel active metabolites. In addition to genotype variations, clinical factors such as diabetes have been shown to alter PK profile of clopidogrel, leading to less potent platelet inhibition in diabetic patients (Angiolillo et al. 2014).

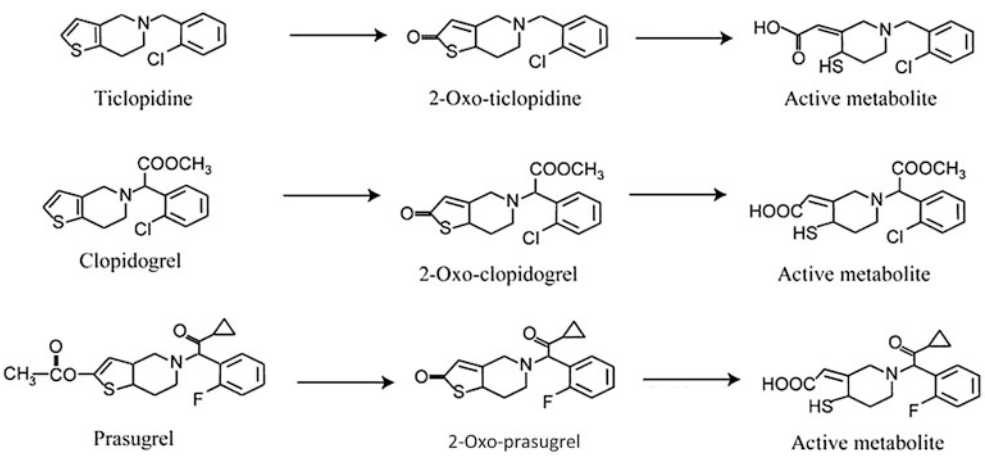

Fig. 4 Thienopyridine active metabolites

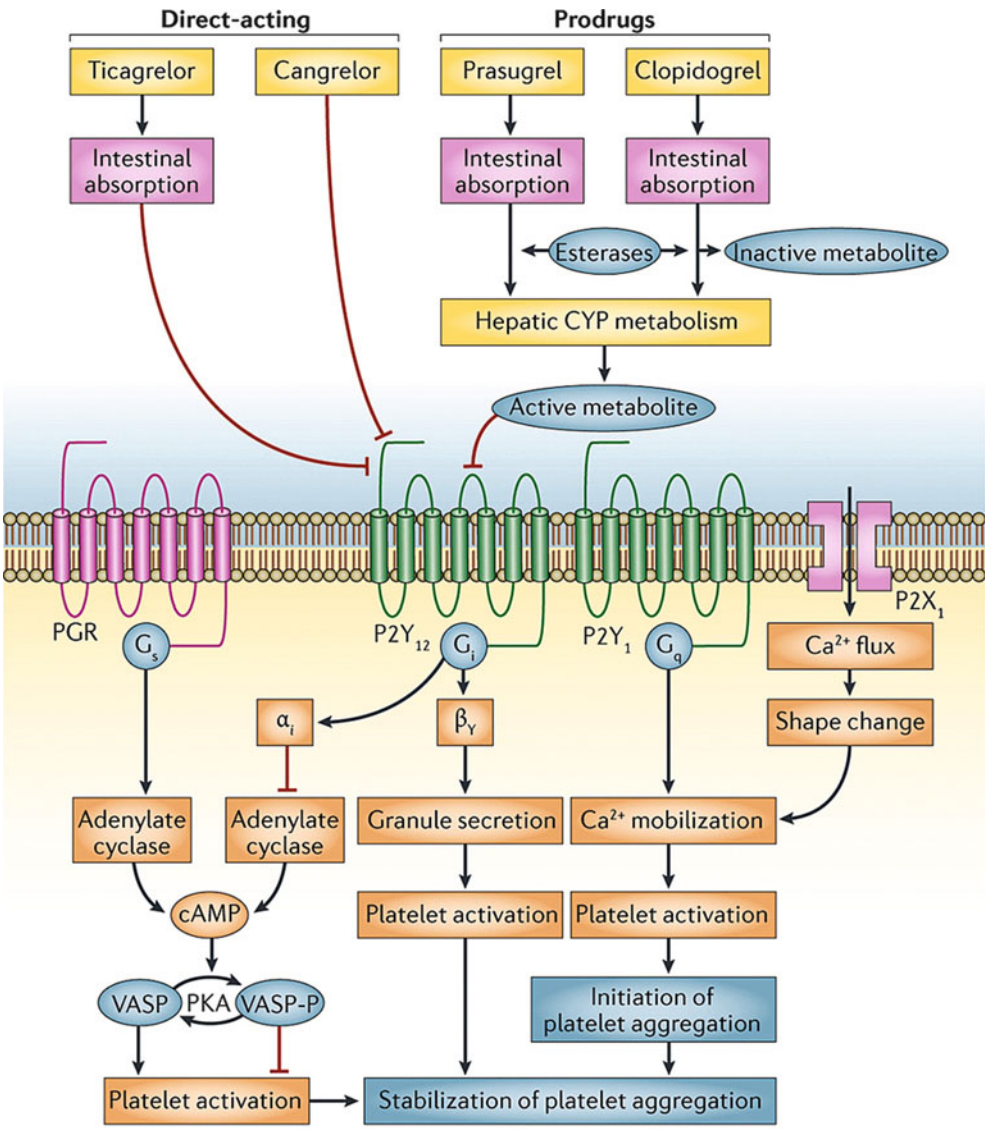

Fig. 5 Mechanisms of action of $P2Y_{12}$ inhibitors. After intestinal absorption, ~85 % of the oral prodrug clopidogrel is hydrolyzed in the blood by esterases to an inactive metabolite; the remaining 15 % is converted to the active metabolite by hepatic CYP isoenzymes, in a two-step oxidation process. By contrast, prasugrel is oxidized more effectively to its active metabolite via a single CYP-dependent step. Cangrelor and ticagrelor do not require hepatic metabolism for pharmacodynamic activity. Ticagrelor is an oral agent that reversibly inhibits platelet activation by allosteric modulation of the $P2Y_{12}$ receptor. Cangrelor is an intravenous ATP analogue, with no need for intestinal absorption, and reversibly inhibits the $P2Y_{12}$ inhibitor (Franchi and Angiolillo 2015). *AC* adenylyl cyclase, *CYP* cytochrome P450, *PGR* prostaglandin receptor, *PKA* cAMP-dependent protein kinase A, *VASP* vasodilator-stimulated phosphoprotein, *P* phosphorylated. Reprinted with permission from Fabiana Rollini, Francesco Franchi, and Dominick J. Angiolillo, Nature Reviews Cardiology 2016 (Rollini et al. 2016), by permission of Nature Reviews Cardiology

As a result of the variability of active metabolite generation, a significant proportion of patients (15–40 %) were described to have inadequate response to standard clopidogrel doses, exposing them to a higher risk for recurrent thrombotic events (Yusuf et al. 2001; Steinhubl et al. 2002; Chen et al. 2005; Angiolillo et al. 2007a; Stone et al. 2013; Tantry et al. 2013; Aradi et al. 2014; Brar et al. 2011).

In addition to the highly variable antiplatelet effect provided by the drug, another important limitation is the relatively slow onset of action of clopidogrel. These limitations urged the development of more potent $P2Y_{12}$ inhibitors with a faster onset of action and more predictable antiplatelet effect.

Prasugrel

The third-generation thienopyridine prasugrel (see Fig. 3) was approved by the FDA in 2009. It is an orally administered prodrug, which requires a two-step metabolism with oxidation to 2-oxo-prasugrel being performed by intestinal esterases just upon absorption, followed by a single hepatic step involving hepatic CYP to generate the active metabolite (see Figs. 4 and 5, Table 1). Although the molar potency of the respective active metabolites of prasugrel and clopidogrel is identical, the metabolic conversion of prasugrel is more efficient, resulting in higher in vivo availability (Sugidachi et al. 2007). These pharmacokinetic properties of prasugrel translate into a faster onset of action, on average more potent platelet inhibitory effect and significantly lower interindividual variability in the active metabolite levels and regarding platelet inhibition compared to clopidogrel (see Table 1) (Franchi and Angiolillo 2015; Wiviott et al. 2007b).

Platelet inhibition is observed 15–30 min after administration of a loading dose of 60 mg, and maximum 60–75 % platelet inhibition is usually achieved within 2–4 h (see Table 1) (Wallentin 2009; Jernberg et al. 2006; Wallentin et al. 2008; Ernest et al. 2008; Asai et al. 2006; Brandt et al. 2007; Jakubowski et al. 2006). After discontinuation of treatment, platelet aggregation returns to pretreatment levels within 7–10 days (Wallentin 2009; Asai et al. 2006; Jakubowski et al. 2006, 2007a; Matsushima et al. 2006). Platelet inhibition is dose dependent, with maximal levels of inhibition (60–75 %) occurring after administration at doses greater than 30 mg (Wallentin 2009; Jernberg et al. 2006; Jakubowski et al. 2007a, b). The C_{max} of prasugrel's pharmacologically active metabolite (see Fig. 3), R-138727, is achieved approximately 30 min after oral administration, with a dose-dependent exposure to prasugrel metabolites in humans (Farid et al. 2008, 2010; Asai et al. 2006; Matsushima et al. 2006). Following a 60-mg loading dose and a 10-mg maintenance dose, the C_{max} values for the active metabolite are about 453 ng/mL and 56 ng/mL, respectively (Farid et al. 2010). Approximately 68 % of the agent is excreted in the urine and 27 % in the feces in the form of inactive metabolites over a period of 10 days, indicating that urinary excretion is the major pathway for the elimination of inactive prasugrel metabolites (Farid et al. 2010).

After oral administration, prasugrel is rapidly and nearly completely (>80 %) absorbed from the intestine (Farid et al. 2007). Prasugrel, like ticlopidine and clopidogrel, is a prodrug and is extensively metabolized. The hydrolysis of prasugrel is mediated by hCE2, an intestinal enzyme, and forms the thiolactone R-95913 through keto-enol tautomerism. R-95913 is metabolized to prasugrel's active metabolite, R-138727 (see Fig. 4). At least half of the dose is rapidly converted to its active metabolite (Farid et al. 2010). Four CYPs are capable of forming prasugrel active metabolite from its thiolactone precursor (CYP3A4, CYP2B6, CYP2C9, CYP2C19) (Rehmel et al. 2006). Administration of maintenance doses or loading doses of prasugrel results in a significantly more rapid onset and more consistent platelet inhibition than observed with clopidogrel in patients with coronary artery disease (Jernberg et al. 2006; Wallentin 2009; Wallentin et al. 2008; Wiviott et al. 2007b; Payne et al. 2007; Brandt et al. 2007; Jakubowski et al. 2007a, b; Weerakkody et al. 2007). In two vulnerable patient groups, i.e., in those weighing less than 60 kg and those above 75 years, the excessive platelet inhibitory effect of prasugrel led to higher risk for bleeding mandating a dose reduction in maintenance dose to 5 mg. Prasugrel is contraindicated in patients with prior history of transient ischemic attack or stroke.

In order to reduce bleeding risk especially in patients in urgent need of surgery, the reversible, direct-acting $P2Y_{12}$ inhibitors ticagrelor and cangrelor were brought into market.

Ticagrelor

Ticagrelor (see Fig. 2) is a noncompetitive, direct-acting $P2Y_{12}$ inhibitor that reversibly binds to the $P2Y_{12}$ receptor (see Figs. 2 and 5). In contrast to clopidogrel and prasugrel, it does not require metabolic activation (see Table 1).

This first-in-class cyclopentyltriazolopyrimidine is an orally administered antiplatelet agent that directly inhibits the $P2Y_{12}$ receptor through allosteric modulation (see Figs. 2 and 5) (Ferreiro and Angiolillo 2012; Franchi and Angiolillo 2015). Unlike the active metabolites of thienopyridines, ticagrelor does not directly block the ADP-binding site; instead, the drug reversibly binds to a distinct site on the $P2Y_{12}$ receptor and prevents ADP from activating the $P2Y_{12}$ pathway, in a noncompetitive fashion (see Fig. 2) (van Giezen et al. 2009; Rollini et al. 2016). As a result, the

$P2Y_{12}$ receptor becomes nonfunctional (locked in inactive state) without losing the capability of ADP binding. Ticagrelor received regulatory approval in Europe and in the United States for the reduction of thrombotic events in patients with acute coronary syndromes in 2011. Due to the reversible nature and the plasma half-life of 8–12 h, it requires a twice-daily dosing regimen (see Table 1) (Teng et al. 2010; Gurbel et al. 2009). Standard loading dose is 180 mg, and standard maintenance dose is 90 mg twice daily.

Ticagrelor is rapidly absorbed with a median time to peak concentration (T_{max}) of 2 h. Although it is a direct-acting agent, approximately 30–40 % of its antiplatelet effects are attributed to an active and approximately equipotent metabolite (AR-C124910XX) generated by hepatic CYP3A4 and CYP3A5 (Teng et al. 2010). After absorption, ticagrelor and AR-C124910XX are highly plasma protein-bound (>99.8 %) (Sillen et al. 2011; Teng et al. 2010). Both exhibit predictable linear pharmacodynamics in patients with atherosclerosis, stable coronary disease, and acute coronary syndromes (Dobesh and Oestreich 2014; Husted et al. 2006, 2012; Teng et al. 2010; Storey et al. 2007). After administration of radiolabeled ticagrelor, mean radioactivity recovery was 58 % in feces and 27 % in urine. However, levels of unchanged ticagrelor and AR-C124910XX in urine were less than 0.05 %, suggesting that renal excretion plays a minor role in the elimination of ticagrelor (Dobesh and Oestreich 2014; Teng et al. 2010).

Ticagrelor achieves rapid (approximately 2 h), dose-dependent, and near-complete (IPA_{MD} ~ 93 %) inhibition of ADP-induced platelet aggregation (see Table 1) that is similar to the potency of prasugrel (Teng and Butler 2010). Consistent with its noncompetitive and reversible mode of $P2Y_{12}$ inhibition, the rate of offset is significantly faster as compared with prasugrel; however, due to the more potent platelet inhibitory effect, the offset is comparable to that of clopidogrel (Gurbel et al. 2009). After discontinuation of treatment, plasma concentrations start to decline after approximately 12 h, and platelet aggregation returns to pretreatment values after 5–7 days (Husted et al. 2006; Teng and Butler 2010; Storey et al. 2010; Gurbel et al. 2009). However, like thienopyridines, ticagrelor is only available orally.

Unlike thienopyridines of which the only known target receptor is $P2Y_{12}$, ticagrelor also blocks equilibrative nucleoside transporter 1 (ENT-1) that is a plasmalemmal transport protein transporting nucleoside substrates such as adenosine. ENT-1 is frequently expressed in many cells, such as on red blood cells. ENT-1 rapidly uptakes adenosine from the plasma that may undergo phosphorylation to form ATP or degradation to uric acid intracellularly. By blocking ENT-1, ticagrelor increases plasma adenosine levels. Although it is thought that elevated plasma levels of adenosine is responsible for the dyspnea after ticagrelor treatment, dipyridamole is even a more potent inhibitor of adenosine reuptake but does not cause dyspnea (Cattaneo and Faioni 2012). Therefore, dyspnea, a common side effect of ticagrelor treatment, is of unknown mechanism.

Cangrelor

All antiplatelet agents discussed so far are limited by their oral distribution, bearing the risk of incomplete absorption in the setting of acute myocardial infarction. Moreover, the currently available data indicate a drug-drug interaction between morphine and oral $P2Y_{12}$ receptor inhibitors, when administered together. First, the CRUSADE registry revealed higher rates of adverse clinical outcomes in non-ST-segment elevation acute coronary syndrome patients treated with clopidogrel who received i.v. morphine, when compared to those who did not (Meine et al. 2005; Kubica et al. 2016). In the ATLANTIC study population, early in-ambulance administration of ticagrelor in patients with ST-segment elevation myocardial infarction improved coronary reperfusion only in patients not receiving morphine (Montalescot et al. 2014). These findings are in line with pharmacodynamics studies (Parodi et al. 2013, 2014, 2015), suggesting that the onset of action of prasugrel and ticagrelor may be delayed by coadministration of morphine in ST-segment elevation myocardial infarction patients (Kubica et al. 2016). Recently, the first randomized, double-blind, placebo-controlled trial featuring three methods of platelet reactivity assessment (vasodilator-stimulated phosphoprotein phosphorylation assay, multiple electrode aggregometry, and VerifyNow) revealed that morphine significantly delays and attenuates ticagrelor exposure and action in patients with myocardial infarction (Kubica et al. 2016). Intravenous administration of $P2Y_{12}$ inhibitors might overcome these shortcomings.

The first and only $P2Y_{12}$ inhibitor with intravenous administration option is cangrelor, that is, an ATP analogue in structure (2-trifluoropropylthio,N-(2-(methylthio)ethyl)-ß,γ-dichloromethylene ATP; see Fig. 3). It was introduced and approved by the FDA in June 2015 for reducing thrombotic events in patients undergoing PCI. As a major advantage compared with oral $P2Y_{12}$ inhibitors, it achieves very potent platelet inhibition and reaches steady-state conditions within a few minutes of initiating administration (see Table 1) (Franchi and Angiolillo 2015; Franchi et al. 2013; Angiolillo et al. 2012b). Cangrelor is an intravenous adenosine triphosphate analogue that directly and reversibly binds to the $P2Y_{12}$ inhibitor in a dose-dependent manner without requiring metabolization (see Fig. 5, Table 1) (Franchi et al. 2013; Angiolillo et al. 2012b). Although the binding site of cangrelor at the $P2Y_{12}$ receptor level is not clearly defined,

cangrelor is associated with high levels of receptor occupancy, preventing ADP signaling (Rollini et al. 2016; van Giezen and Humphries 2005). The modifications in the ATP molecule provide cangrelor with a great affinity for the $P2Y_{12}$ receptor and a higher resistance to ectonucleotidases (Ferreiro et al. 2009). Cangrelor shows a rapid onset of action (2 min) when administration is started with a bolus dose and exhibits a linear dose-response pharmacokinetic profile that leads to very stable pharmacodynamic effects (Franchi and Angiolillo 2015). It also exerts a dose-dependent effect on platelet inhibition with infusions up to 4 μg/kg/min, achieving a high degree (>80 %) of platelet inhibition following 5 and 20 μmol/L ADP stimuli (Storey et al. 2001; Ferreiro et al. 2009; Franchi et al. 2013; Angiolillo et al. 2012b). After discontinuation of infusion, cangrelor shows a very rapid offset of action due to its very short half-life (3–5 min) due to rapid degradation through dephosphorylation, with platelet aggregation returning to pretreatment values within 30–60 min (Franchi and Angiolillo 2015; Franchi et al. 2013; Angiolillo et al. 2012b). These pharmacodynamic properties seem optimal for patients in need of rapid platelet inhibition who are unable to intake oral drugs. Cangrelor might also be a good antiplatelet treatment option for ACS patients requiring CABG surgery. The BRIDGE trial was a pharmacodynamic trial conducted in 210 patients (50 % ACS patients) treated with a thienopyridine and requiring surgery. Cangrelor was associated with high and stable levels of platelet inhibition, with an extremely rapid offset of action after discontinuation before surgery and without increased risk of major bleeding or adverse events before or during CABG surgery (Rollini et al. 2016; Angiolillo et al. 2012a).

An important consideration for the use of cangrelor is how to switch from the intravenous agent to oral $P2Y_{12}$ inhibitors. A pharmacodynamic investigation conducted in healthy volunteers showed that concomitant administration of clopidogrel and cangrelor infusion leads to inadequate clopidogrel-induced antiplatelet effects, because clopidogrel does not bind to the $P2Y_{12}$ receptor while the receptor is occupied by cangrelor (Rollini et al. 2016; Steinhubl et al. 2008). However, clopidogrel-induced antiplatelet effects were not affected when clopidogrel was administered after a cangrelor infusion, given the very rapid offset of action of cangrelor (Steinhubl et al. 2008; Rollini et al. 2016). The transition from cangrelor to prasugrel was associated with transient recovery of platelet reactivity, particularly within 1 h after cangrelor discontinuation, which was attenuated when prasugrel was administered 30 min before stopping cangrelor infusion (Rollini et al. 2016; Schneider et al. 2015). In contrast, a pharmacodynamic study showed that the pharmacodynamic effects of ticagrelor and cangrelor are consistently preserved independently from the sequence in which the drugs are administered. Therefore, ticagrelor can be administered before, during, or after cangrelor infusion without pharmacodynamic interaction (Schneider et al. 2014; Rollini et al. 2016). With this regards, ticagrelor may be beneficial given the distinct $P2Y_{12}$ receptor binding site leading to lack of competition when both drugs are present in the plasma.

Take-Home Messages

- Thienopyridine-type $P2Y_{12}$ inhibitors, such as ticlopidine, clopidogrel, and prasugrel, are prodrugs that require hepatic metabolization and conversion to a pharmacologically active metabolite to exert their antiplatelet effect. Their active metabolites covalently and irreversibly bind to the $P2Y_{12}$ receptor for the entire life span of the platelet; therefore, the offset of the antiplatelet effect is only possible by formation of new platelets.
- Clopidogrel is widely used in various cerebrovascular patients to prevent and treat recurrent thrombotic complications. An important limitation of the drug, however, is the highly variable active metabolite formation leading to unpredictable platelet reactivity inhibition after fixed dosing. Patients with inappropriate platelet reactivity inhibition are exposed to higher risk for stent thrombosis, myocardial infarction, and mortality.
- Prasugrel provides more potent and more predictable antiplatelet effect with a quicker onset of action as compared to clopidogrel, although their active metabolites have similar $P2Y_{12}$ inhibitory effect at equimolar concentrations. The more potent and more predictable antiplatelet effect translated into improved clinical outcomes in patients with ACS undergoing percutaneous coronary intervention, albeit at the expense of an increased bleeding risk.
- Ticagrelor is a cyclopentyltriazolopyrimidine-type, reversible binding, direct-acting $P2Y_{12}$ inhibitor, not requiring metabolic activation. It also provides a rapid and predictable $P2Y_{12}$ inhibition with similar safety and efficacy profile as prasugrel. Importantly, ticagrelor also inhibits adenosine reuptake by blocking ENT-1, causing an elevated level of adenosine with unknown clinical consequences.
- Cangrelor is an intravenous $P2Y_{12}$ inhibitor, with a unique PK profile: it achieves very potent platelet inhibition and reaches steady-state conditions within a few minutes of initiating administration and shows a very rapid offset of action after discontinuation.

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Glycoprotein IIb/IIIa Antagonists

Madhan Shanmugasundaram and David J. Moliterno

Abstract

Patients with acute coronary syndrome and who undergo percutaneous coronary intervention are at a high risk for recurrent adverse cardiovascular events. There is a continuous search for an ideal antiplatelet medication that has a favorable risk benefit profile with a quick onset of peak action. Glycoprotein IIb/IIIa inhibitors (GP IIb/IIIa) were introduced with that precise idea in mind, that it is an intravenous antiplatelet agent with a rapid onset of action, which can be used to achieve maximal antiplatelet activity in patients with acute coronary syndrome (ACS) and those undergoing percutaneous coronary intervention (PCI). Abciximab, eptifibatide, and tirofiban are the three available intravenous GP IIb/IIIa inhibitors of which the latter two are small molecules. Initial trials that examined these agents were done in the balloon angioplasty era when $P2Y_{12}$ receptor blocker use was not routine. These trials demonstrated a significant reduction in the composite end point of death, myocardial infarction (MI), and urgent target vessel revascularization predominantly driven by a reduction in recurrent MI that included periprocedural events. There is a definite increase in major and minor bleeding with these agents which needs to be weighed against the potential benefits before initiating the drug. More contemporary trials that were done on patients who were adequately treated with $P2Y_{12}$ inhibitors failed to recreate the initial results, but nevertheless there was still a significant reduction in ischemic events. There is still a role for these agents in patients with high-risk non-ST segment elevation myocardial infarction and ST segment elevation myocardial infarction with heavy thrombus burden, particularly if they have not been adequately pretreated with $P2Y_{12}$ inhibitors.

Platelet Biology

It is well established that platelets play a critical role in normal hemostasis and pathogenesis of thromboembolic disease. Beyond being the primary cellular component of arterial thrombosis, platelets are involved in fibrin formation, inflammation, and fibrinolysis. When an atherosclerotic plaque ruptures, the exposed subendothelial matrix induces platelet activation and aggregation in three phases, initiation, extension, and perpetuation phase, resulting in the formation of platelet plug or white thrombus (Davi and Patrono 2007). In the initiation phase, circulating platelets bind to the exposed subendothelial matrix at the site of endothelial injury via GP Ib-IX receptor complex that binds to the von Willebrand factor (vWF). This triggers platelet activation and release of various platelet-activating agonists such as thromboxane A2 (TxA2), thrombin, serotonin, and adenosine diphosphate (ADP). TxA2 and ADP activate the platelets directly through G-protein-coupled receptors resulting in platelet aggregation (Hughes and Pfaff 1998).

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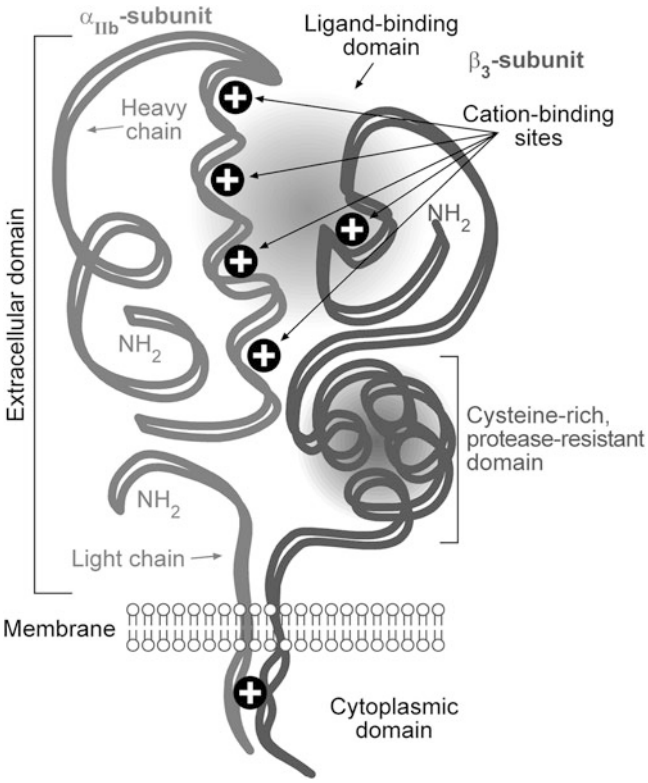

Fig. 1 Structure of the GP IIb/IIIa receptor. Reproduced with permission from Topol EJ, Byzova TV, Plow EF. Platelet GP IIb/IIIa blockers. Lancet 1999;353:227–231

The extension phase thus recruits more platelets to the site of endothelial damage. Platelet aggregation is predominantly mediated by glycoprotein (GP) IIb/IIIa receptors. GP IIb/IIIa receptors undergo a conformational change in activated platelets which allows them to bind with specific ligands such as fibrinogen, fibronectin, and von Willebrand factor (Lefkovits et al. 1995). This step is critical in the formation of platelet plug, thus making the GP IIb/IIIa receptors an attractive pharmaceutical target as it represents the final common pathway in the formation of white thrombus (Angiolillo et al. 2010). In the perpetuation phase the platelet-rich thrombus and coagulation cascades interact with one another by a series of complex mechanisms resulting in the formation of stable platelet-fibrin-rich plug. It is estimated that there are approximately 80,000 GP IIb/IIIa receptors on the surface of each platelet (Wagner et al. 1996). Figure 1 shows the structure of a GP IIb/IIIa receptor.

Glycoprotein IIb/IIIa Inhibitors

It has been shown by in vitro studies that intravenous GP IIb/IIIa inhibitors block 80–90 % of ADP-induced platelet aggregation in a dose-dependent fashion compared to 10 % by aspirin and 30–50 % for thienopyridines. There are three GP IIb/IIIa inhibitors available for routine clinical use: abciximab, eptifibatide, and tirofiban.

Abciximab

Abciximab (ReoPro®) was the first GP IIb/IIIa inhibitor to be approved by FDA for clinical use in 1997. It is an antibody against the GP IIb/IIIa receptor which rapidly and firmly binds the IIb/IIIa receptor (Coller et al. 1983). As such, abciximab has a very short plasma half-life but a long biologic half-life (up to 48 h) (Schror and Weber 2003). Because abciximab near irreversibly binds the receptor, there are no reversal agents. Platelet transfusions provide some, but limited, reversal effect since abciximab can continue to bind to the newly infused platelets (Webb et al. 2011). The recommended bolus dose is 0.25 mg/kg followed by 0.125 mcg/kg/min infusion for up to 12 h in patients with acute coronary syndrome with planned invasive strategy. Bleeding is the most common side effect as reported in various placebo controlled trials (EPIC Investigators. 1994). Although thrombocytopenia is reported in 5–6 % of patients in early abciximab trials, clinically significant thrombocytopenia (platelet count $< 100{,}000/cc^3$) occurred in 2 % of patients. Even though the incidence of thrombocytopenia is higher with abciximab compared to the other GP IIb/IIIa inhibitors, there appears to be no significant increase in bleeding.

Eptifibatide

Eptifibatide (Integrilin®) is a small-molecule cyclic heptapeptide with high specificity for the GP IIb/IIIa receptor but low affinity. Hence it disassociated rapidly from the receptor and has a short biologic half-life (Scarborough et al. 1999). It is cleared mostly by kidneys; hence dose adjustment is necessary based on creatinine clearance and is contraindicated in hemodialysis patients (Alton et al. 1998). It has been shown that in 10–15 min after the bolus dose, there is nearly complete inhibition of ADP-induced platelet aggregation, and effect persists for 2–4 h after the infusion is stopped. The dose of eptifibatide is 180 μg/kg bolus followed by a maintenance infusion of 2 μg/kg/min for 18–24 h. It was shown that the pharmacodynamics of eptifibatide was significantly altered by anticoagulants that chelate calcium, resulting in overestimation of the in vivo effects of this agent. This resulted in the use of lower doses of eptifibatide in some earlier trials which in turn resulted in lower efficacy (Gilchrist et al. 2001). The dosing regimen is different when used in the percutaneous coronary intervention (PCI) setting where a double bolus (180 mcg/kg) 10 min apart followed by

infusion is recommended. This was based on the findings of ESPRIT trial which was terminated prematurely due to superior results compared to placebo (ESPRIT Investigators. 2000). Even though bleeding is the major side effect, it was shown that TIMI major bleeding rates were not statistically different compared to placebo. Thrombocytopenia is also uncommon with eptifibatide.

Tirofiban

Tirofiban (Aggrastat®) is a small-molecule, non-peptide tyrosine derivative that causes reversible GP IIb/IIIa inhibition. Similar to eptifibatide, it also has a high specificity for the GP receptor, but the platelet-bound half-life is extremely short (Barrett et al. 1994). The plasma half-life is about 2 h and platelet function returns to baseline within 6–8 h after cessation of the drug. It is cleared mostly by the kidneys; hence dose adjustment for advanced renal disease is required, and it is contraindicated in patients on dialysis.

Currently recommended dosing is 25 mcg/kg bolus followed by infusion at 0.15 mcg/kg/min for up to 18 h. Earlier tirofiban trials however used a lower dose that resulted in inferior results, but the current dosing recommendations were based on pharmacodynamic trials that established superior platelet inhibition with the higher bolus dose. Bleeding and thrombocytopenia are the most commonly reported adverse events with tirofiban with rates similar to those reported with eptifibatide. Important pharmacokinetic properties of the GP IIb/IIIa inhibitors are summarized in Table 1.

Table 1 Pharmacology of GP IIb/IIIa inhibitors

Characteristic	Abciximab	Eptifibatide	Tirofiban
Size	Large molecule	Small molecule	Small molecule
Nature of drug	Monoclonal antibody	Heptapeptide	Non-peptide
Plasma half-life	10 min	2.5 h	2 h
Platelet half-life	Hours	Seconds	Seconds
Dose	Bolus: 0.25 mg/kg Maintenance: 0.125 mcg/kg/min for 12 h	Bolus: 180 mcg/kg × 2 Maintenance: 2 mcg/kg/min for 12–18 h	Bolus: 25 mcg/kg Maintenance: 0.15 mcg/kg/min for 12–18 h
Time taken for return of platelet function after cessation of drug	24 h	4 h	4 h
Clearance	Spleen	Renal	Renal
Reversal	Platelets infusion	None	None

Role of GP IIb/IIIa Inhibitors

Unstable Angina and Non-ST Elevation Myocardial Infarction

Glycoprotein IIb/IIIa inhibitors were introduced to address the problem of increased major adverse cardiovascular events (MACE) in patients presenting with acute coronary syndrome (ACS) and hence form a class of well-studied medication in this cohort of patients. This is a heterogeneous group to begin with, and not all patients had routine early invasive approach which makes it hard to generalize any data to the contemporary ACS population, but there was still a 10 % relative reduction in the 30-day incidence of death or MI. Most of the benefit from the GP IIb/IIIa inhibitors is from reduction of recurrent MI and periprocedural infarction. It should also be noted that most of these GP IIb/IIIa inhibitor trials were performed in the era of balloon angioplasty and no routine use of thienopyridines.

One of the first major trials examining the use of GP IIb/IIIa inhibitors in unstable angina patients undergoing balloon angioplasty was the EPIC trial. It showed a significant reduction of death, MI, recurrent ischemia, or failed revascularization in patients randomized to abciximab therapy (1994). ISAR REACT 2 trial randomized over 2000 patients with non-ST elevation MI (NSTEMI) undergoing invasive treatment to abciximab or placebo. It showed a significant reduction in major adverse cardiovascular events (death, MI, or urgent revascularization) at 30 days in patients who received abciximab (Kastrati et al. 2006). This trial included patients who were given a loading dose of clopidogrel (600 mg) hence proving that there is role for GP IIb/IIIa inhibitors in these patients. No significant difference in bleeding was noted in either of these two studies. Glycoprotein IIb/IIIa inhibitors were tested as part of upstream therapy in patients planned for early invasive strategy. CAPTURE trial randomized patients with refractory unstable angina (recurrent myocardial ischemia on maximal medical therapy including heparin and nitrates) who were undergoing balloon angioplasty to abciximab or placebo. This trial again showed a significant reduction in MACE at 30 days (CAPTURE Investigators. 1997). The GUSTO IV ACS trial was unique in the fact that it examined the use of abciximab in patients who were not undergoing early intervention. It showed no significant difference in MI or death at 30 days and hence concluded that upstream use of abciximab in patients undergoing medical therapy only for NSTEMI may not be beneficial, thus the class III recommendation in this clinical scenario (Simoons 2001).

PRISM trial showed that in patients with unstable angina receiving heparin and aspirin, tirofiban use reduced death, MI, and refractory ischemia at 48 h compared to placebo at

the expense of increased bleeding (PRISM Investigators. 1998a). PRISM PLUS trial compared the use of tirofiban alone to heparin alone and combination of tirofiban and heparin in patients with unstable angina and NSTEMI. This trial was terminated early due to increased mortality in patients randomized to tirofiban alone but showed a significant reduction in death, MI, or refractory ischemia within 7 days in patients on combination therapy (tirofiban and heparin). About one third of patients underwent balloon angioplasty and the rest were treated conservatively (1998c). ADVANCE trial examined the use of high bolus dose tirofiban in patients with NSTEMI who were pretreated with clopidogrel, undergoing high-risk PCI, and demonstrated a significant reduction of MACE at 180 days (Valgimigli et al. 2004).

The PURSUIT trial was one of the larger trials (over 10,000 patients) to examine the use of eptifibatide in patients presenting with unstable angina or NSTEMI. It showed a significant reduction in death or MI up to 30 days in patients who received eptifibatide at the expense of increased bleeding (PURSUIT Investigators. 1998b). The EARLY ACS trial tested the hypothesis of upstream eptifibatide use in patients with NSTEMI who were planned to undergo PCI. This study showed no significant difference in ischemic outcomes (death or MI) in patients who received the drug early (12 h before angiography) compared to the ones who received it during angiography, and also early GP IIb/IIIa inhibitor use was associated with more bleeding (Giugliano et al. 2009). However it should be noted that EARLY ACS trial was conducted in an era of frequent use of stents and routine early angiography compared to PURSUIT trial. A meta-analysis by Tricoci et al. showed an 11 % reduction in ischemic end points (death or MI) at 30 days with upstream use of small-molecule GP IIb/IIIa inhibitors in patients with NSTEMI, but a 23 % increase in major bleeding was seen in these patients (Tricoci et al. 2011). These trials clearly demonstrate that routine upstream use of GP IIb/IIIa inhibitors in ACS patients may not be beneficial due to the modest ischemic benefit being outweighed by higher bleeding risk. A recently published Cochrane review of GP IIb/IIIA inhibitors that included 60 randomized trials (over 60,000 patients) concluded that with downstream use (during PCI), there was a significant reduction at death or MI at 30 days with significantly increased major bleeding risk (odds ratio 1.39, CI 1.21–1.61) in patients receiving GP IIb/IIIa inhibitors. With upstream use there was a slight decrease in death or MI at 30 days with increased major bleeding (odds ratio 1.29, CI 1.14–1.45). The number needed to treat (death or MI) was 42, and the number needed to harm (major bleeding) was 125. The benefits of GP IIb/IIIa inhibitors appeared to be homogenous across different population subgroups although less marked for patients pretreated with clopidogrel (Bosch et al. 2013).

In the latest ACC/AHA guidelines for management of patients with non-ST elevation acute coronary syndromes, GP IIb/IIIa inhibitors have received a class I recommendation in patients undergoing early invasive approach if they have not been adequately pretreated with a second antiplatelet drug (clopidogrel, prasugrel, or ticagrelor) and class IIa in patients adequately pretreated with two oral antiplatelets if they have a high-risk presentation (Amsterdam et al. 2014). The European Society of Cardiology (ESC) guidelines assign a class IIa recommendation for GP IIb/IIIa inhibitors use during PCI only for bailout situations or thrombotic complications (Roffi et al. 2016). It is very clear in the guidelines that routine upstream use of GP IIb/IIIa inhibitors is generally discouraged.

ST Elevation Myocardial Infarction

Glycoprotein IIb/IIIa inhibitors have been studied extensively in patients with ST elevation myocardial infarction (STEMI) who underwent either thrombolytic therapy or primary PCI. Primary PCI has been established to be superior to thrombolytic therapy in STEMI, but only a minority of patients in the United States present to a PCI-capable hospital. This paved way for a series of studies examining different pharmacologic strategies aimed at achieving comparable results to primary PCI. Facilitated PCI is a strategy of pharmacologic reperfusion therapy delivered prior to a planned PCI. As a part of this strategy, GP IIb/IIIa inhibitors were combined with different fibrinolytics in various doses to achieve reperfusion, but overall, this was shown to be inferior to pharmaco-invasive approach (thrombolytic therapy administered at a non-PCI hospital with intent of transferring to a PCI hospital for early angiography – within 3–24 h) or primary PCI. In earlier trials such as GUSTO V (Topol 2001) and ASSENT-3 (2001), GP IIb/IIIa inhibitors were combined with thrombolytics and were shown to increase bleeding risk without additional ischemic benefit. It should be noted that pre-procedural thienopyridine use was not routine in these earlier trials. Upstream abciximab use with 600 mg of clopidogrel was tested in BRAVE-3 study where it was shown that there was no difference in the infarct size (Tc-99 m sestamibi SPECT imaging) compared to placebo (Mehilli et al. 2009). A sub-study of APEX AMI (Huber et al. 2010) trial supported the idea of upstream GP IIb/IIIa use in STEMI patients, but this finding was refuted in the larger FINESSE study. In FINESSE trial over 1500 patients with STEMI were randomized to one of the three arms of the study: either half-dose reteplase combined with abciximab or abciximab alone or primary PCI in the background of aspirin and heparin therapy. The study failed to show any difference in ischemic end point, but increased bleeding and transfusion requirements were seen (Ellis et al. 2008).

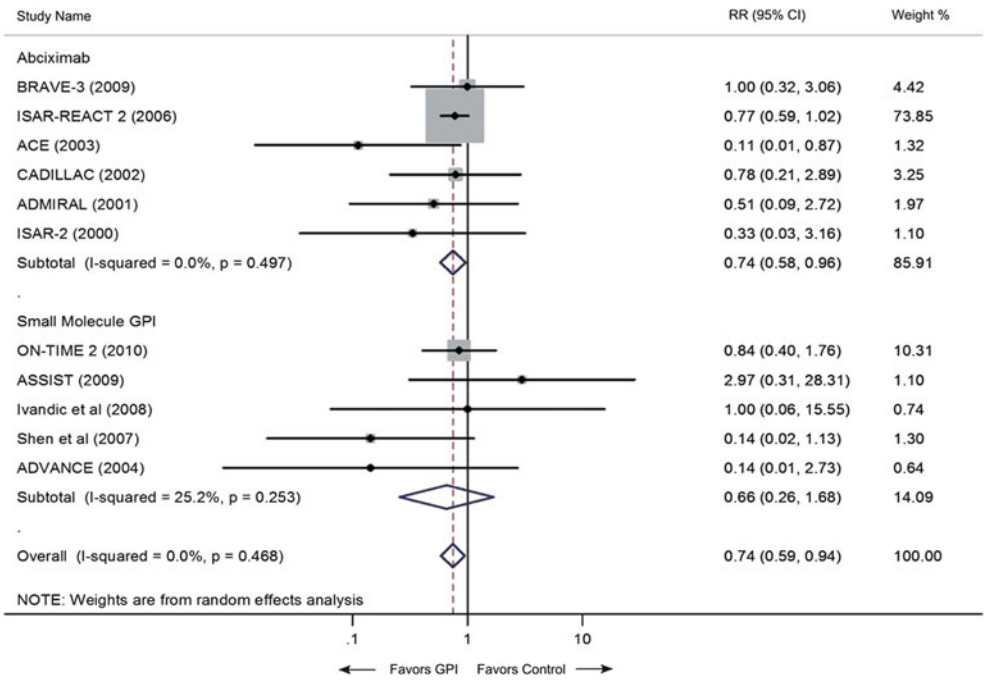

Fig. 2 Risk ratio for myocardial infarction in ACS patients. Reproduced with permission from Winchester, D.E., et al., *Efficacy and safety of unfractionated heparin plus glycoprotein IIb/IIIa inhibitors during revascularization for an acute coronary syndrome: a meta-analysis of randomized trials performed with stents and thienopyridines*. Clin Cardiol, 2012. **35**(2): p. 93–100

Downstream (administered in the catheterization lab at the time of PCI) abciximab use was tested in several small trials in patients undergoing primary PCI and showed some ischemic benefit at the expense of increased bleeding (Brener et al. 1998; Neumann et al. 2000; Montalescot et al. 2001; Stone et al. 2002; Antoniucci et al. 2003). It should however be mentioned that in HORIZONS-AMI trial routine GP IIb/IIIa use with heparin was compared to bivalirudin in patients undergoing primary PCI for STEMI. It was shown that there was no added ischemic benefit from GP IIb/IIIa use; however, there was increased bleeding noted in this group (Stone et al. 2008). A meta-analysis by Dong et al. examined the role of GP IIb/IIIa inhibitors in patients with STEMI undergoing primary PCI after pretreatment with loading dose thienopyridine. They showed that there was no significant difference in 30-day or 6-month death or MI in patients treated with GP IIb/IIIa inhibitors but there was significantly increased bleeding risk (Dong et al. 2013). The Cochrane review also showed slight reduction in 30-day MACE (death, MI, or urgent TVR) with increased major bleeding risk in STEMI patients (Bosch et al. 2013).

A recently published meta-analysis examined the role of GP IIb/IIIa inhibitors in patients with ACS, in the current era of stents and routine thienopyridine use, and the results of this study reflect the practice guidelines. There was a 26 % relative risk reduction in MI at 30 days with GP IIb/IIIa inhibitors use (3.1 % in GP IIb/IIIa inhibitors versus 4.4 % with control, RR = 0.74; 95 % CI 0.59–0.94, $p = 0.014$) (Fig. 1). In regard to safety, it was noted that there was an increase in TIMI minor bleeding, but the incidence of major bleeding at 30 days was 2.5 % with GP IIb/IIIa inhibitors versus 2.1 % with control, hence not different (RR = 1.21; 95 % CI 0.89–1.63, $p = 0.22$) (Fig. 2) (Winchester et al. 2012). In the 2013 ACC/AHA STEMI guidelines, GP IIb/IIIa inhibitors were given a class IIa recommendation in selected patients undergoing primary PCI (heavy thrombus burden and inadequate $P2Y_{12}$ receptor antagonist loading) and class IIb in pre-catheterization setting with no discrimination being made among the three agents (O'Gara et al. 2013). GP IIb/IIIa inhibitors received a class IIa recommendation in the recent ESC STEMI guidelines, as a bailout therapy in the presence of massive thrombus, slow or no-reflow or a thrombotic complication (Steg et al. 2012).

Percutaneous Coronary Intervention

Glycoprotein IIb/IIIa inhibitors were studied in patients undergoing outpatient non-emergent PCI. As previously mentioned most of these trials were done in the era of balloon angioplasty only or bare metal stents and when $P2Y_{12}$ receptor antagonist use was not standard practice. Hence in some initial trials, there was a reduction in MACE events, but a closer look at these trials showed that most of the benefit

was from reduction of recurrent MI, likely related to reduction of periprocedural infarction. Also some of the earlier trials used a different dose of these medications as the appropriate dose that achieved maximal inhibition of platelet aggregation had not been identified. Over 2000 patients who were scheduled to undergo elective PCI were randomized to eptifibatide therapy or placebo in the ESPIRIT trial, which was terminated early due to efficacy. This study showed significant reduction in death, MI, or urgent TVR in patients randomized to eptifibatide therapy.

ISAR REACT trial randomized patients undergoing elective PCI who were preloaded on clopidogrel to abciximab or placebo and demonstrated that addition of GP IIb/IIIa inhibitor did not provide any significant ischemic benefit in this population. This trial is more similar to our current day PCI practice pattern and hence convincingly showed that routine use of GP IIb/IIIa inhibitors for elective PCI in patients preloaded on $P2Y_{12}$ antagonists is not beneficial (Kastrati et al. 2004). Winchester et al. published a meta-analysis of 22 trials that included over 10,000 patients undergoing elective PCI with stents adequately preloaded on $P2Y_{12}$ inhibitors, comparing the utility of GP IIb/IIIa inhibitors. There was a 31 % relative risk reduction in nonfatal MI in patients receiving GP IIb/IIIa inhibitors compared to placebo (5.1 % vs. 8.3 %, RR 0.69, 95 % CI 0.55–0.86, $p = 0.001$) (Fig. 3) with no difference among the type of GP IIb/IIIa inhibitors used. There was no reduction in all-cause mortality with GP IIb/IIIa inhibitors use. Even though there was an increase in TIMI minor bleeding with GP IIb/IIIa inhibitors use 3.0 % versus 1.7 % with control (RR: 1.70, 95 % CI 1.28–2.26, $p < 0.0001$), there was no increase in TIMI major bleeding. The incidence of major bleeding was 1.2 % with GP IIb/IIIa inhibitors versus 0.9 % with placebo (RR 1.37, 95 % CI 0.83–2.25, $p = 0.22$) (Fig. 4) (Winchester et al. 2011). According to the 2011 PCI guidelines, routine use of GP IIb/IIIa inhibitors in patients undergoing elective PCI (treated with heparin and adequately preloaded on clopidogrel) is a class IIb recommendation if adequately pretreated with clopidogrel and class IIa recommendation if not pretreated (Levine et al. 2011) (Fig. 5).

Intracoronary Use of Glycoprotein IIb/IIIa Inhibitors

Optimizing outcomes and minimizing complications in patients undergoing PCI are critical. During PCI there is often distal micro- or macroembolization which contributes to periprocedural MI that has been shown to increase mortality. It was proposed that if GP IIb/IIIa inhibitors could be delivered locally via intracoronary administration, myocardial perfusion could be maintained during PCI.

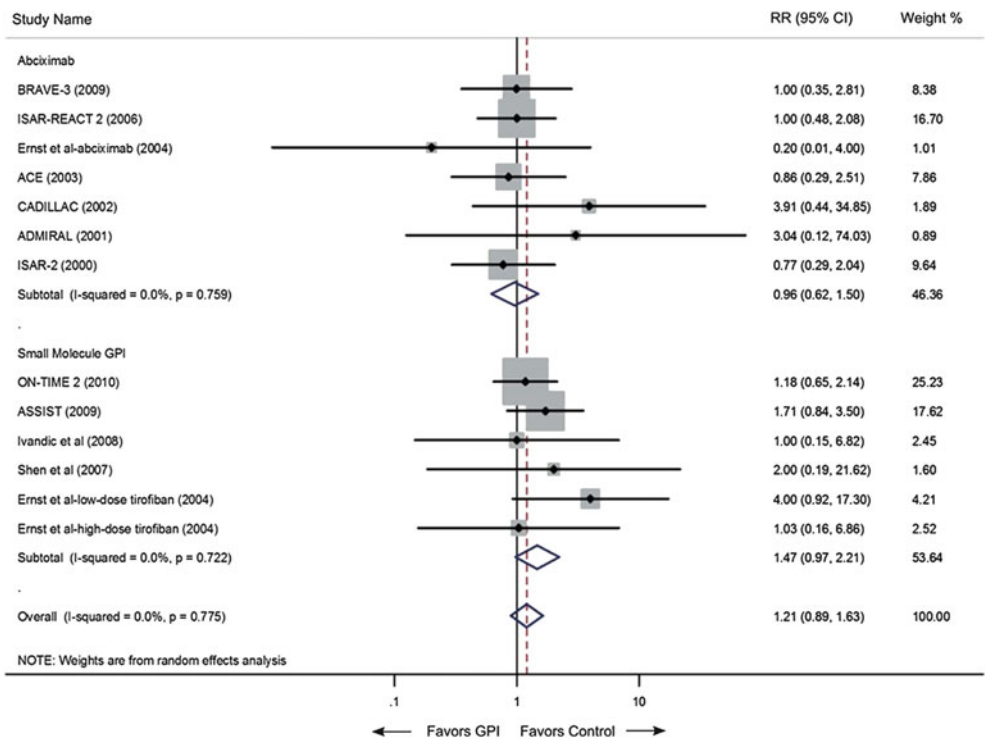

Fig. 3 Risk ratios for major bleeding in ACS patients. Reproduced with permission from Winchester, D.E., et al., *Efficacy and safety of unfractionated heparin plus glycoprotein IIb/IIIa inhibitors during revascularization for an acute coronary syndrome: a meta-analysis of randomized trials performed with stents and thienopyridines*. Clin Cardiol, 2012. 35(2): p. 93–100

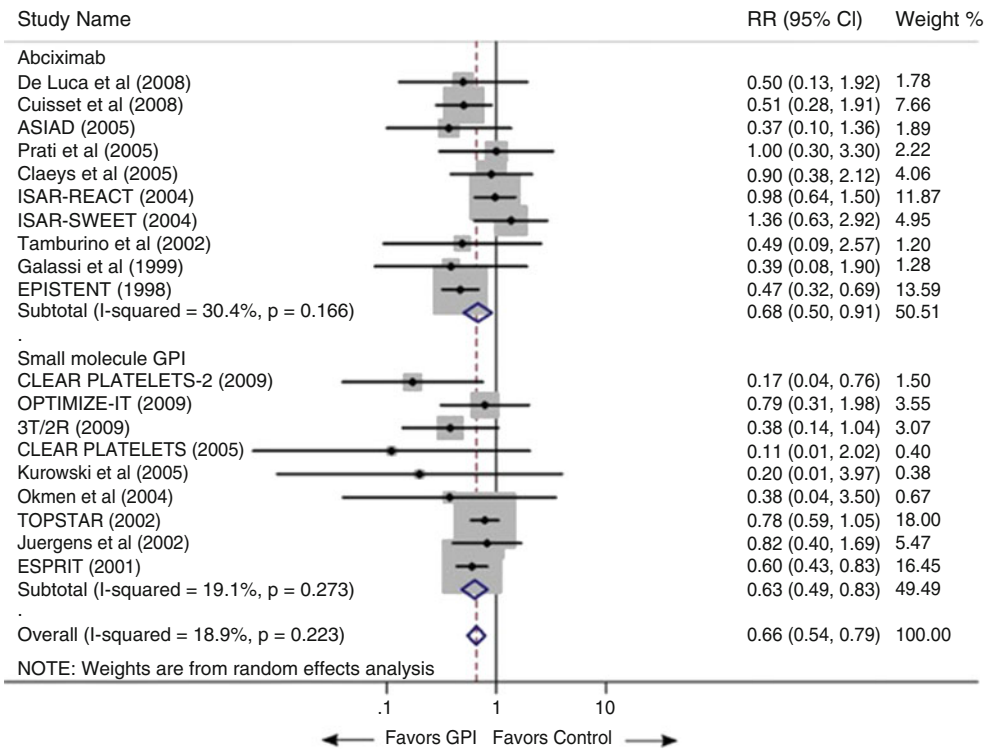

Fig. 4 Risk ratios for myocardial infarction in elective PCI patients. Reproduced with permission from Winchester, D.E., et al., *Efficacy and safety of glycoprotein IIb/IIIa inhibitors during elective coronary revascularization: a meta-analysis of randomized trials performed in the era of stents and thienopyridines*. J Am Coll Cardiol, 2011. 57(10): p. 1190–1199

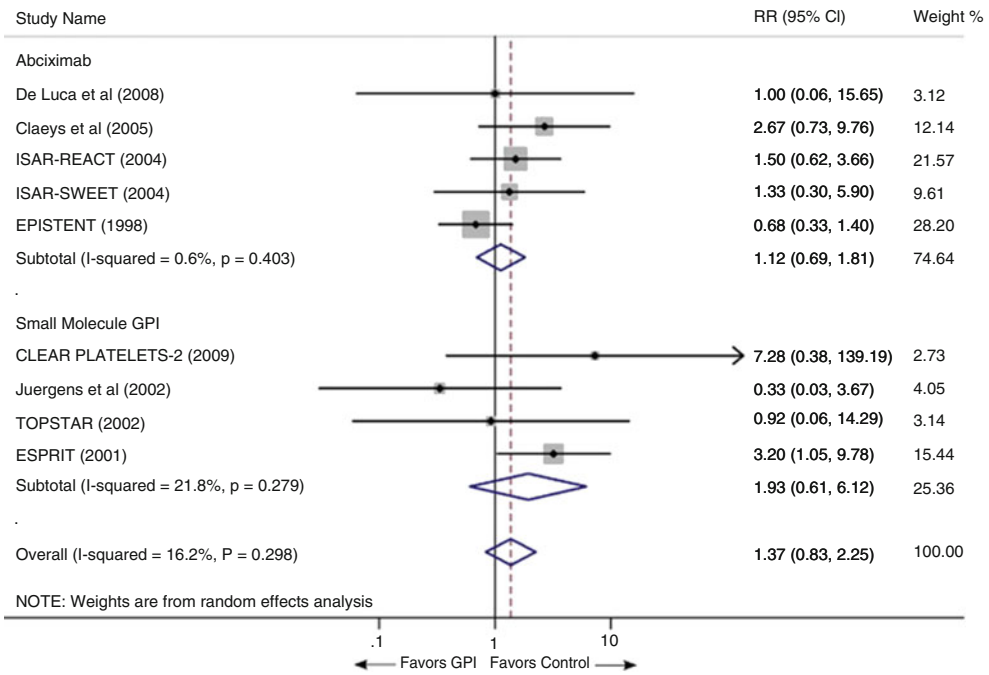

Fig. 5 Risk ratios for major bleeding in elective PCI patients. Reproduced with permission from Winchester, D.E., et al., *Efficacy and safety of glycoprotein IIb/IIIa inhibitors during elective coronary revascularization: a meta-analysis of randomized trials performed in the era of stents and thienopyridines*. J Am Coll Cardiol, 2011. 57(10): p. 1190–1199

Table 2 Trials examining the use of abciximab in different clinical scenarios

	EPISTENT (Investigators 1998)	ISAR REACT 2 (Kastrati et al. 2006)	BRAVE-3 (Mehilli et al. 2009)	CAPTURE (1997)	GUSTO IV ACS (Simoons 2001)
Clinical scenario	Elective or urgent PCI	NSTEMI	STEMI (abciximab vs. reteplase + abciximab)	Unstable angina (refractory)	NSTEMI (placebo vs. 24 h abciximab vs. 48 h abciximab)
Number of patients	2399	2022	800	1265	7800
Timing of drug	Downstream	Downstream	Downstream	Upstream	Upstream
Dose	0.25 mg/kg bolus 0.125 mcg/kg/min for 12 h	0.25 mg/kg bolus 0.125 mcg/kg/min for 12 h	0.25 mg/kg bolus 0.125 mcg/kg/min for 12 h	0.25 mg/kg bolus 0.125 mcg/kg/min for 18–24 h	0.25 mg/kg bolus 0.125 mcg/kg/min for 24–48 h
Other anticoagulants or antiplatelets	Heparin, ASA ± ticlopidine	Heparin, ASA, clopidogrel	Heparin, ASA, clopidogrel	Heparin, ASA ± ticlopidine	Heparin or LMWH, ASA
Invasive treatment arm	100 % PTCA > BMS	100 % DES > BMS ≫ PTCA	100 % BMS ≫ PTCA	100 % PTCA	Conservative therapy only
Primary outcome	Death or MI at 1 year	Death, MI, or urgent TVR	Infarct size (SPECT)	Death, MI, or urgent TVR	Death or MI at 30 days
Results	5.3 % vs. 10.8 % ($p < 0.001$)	8.9 % vs. 11.9 % ($p = 0.03$)	15.7 % vs. 16.6 % (ns)	11.3 % vs. 15.9 % ($p = 0.012$)	8.2 % vs. 8 % (ns) (24 h group) 9.1 % vs. 8 % (ns) (48 h group)

PCI percutaneous coronary intervention, *NSTEMI* non-ST elevation myocardial infarction, downstream: after angiography or at the time of PCI, upstream: at the time of admission or before angiography, *PTCA* percutaneous transluminal coronary angioplasty, *BMS* bare metal stent, *DES* drug-eluting stent, *MI* myocardial infarction, *TVR* target vessel revascularization, *SPECT* single photon emission computed tomography, *ASA* aspirin, *LMWH* low molecular weight heparin

Intracoronary administration of eptifibatide was shown to increase localized platelet GP IIb/IIIa receptor occupancy and microvascular perfusion (measured by corrected TIMI frame count) in patients undergoing PCI for ACS (Deibele et al. 2010). Intracoronary tirofiban was shown to be similar to intravenous bolus and infusion of tirofiban in patients undergoing primary PCI, in a small study, in regard to microvascular perfusion and ST segment resolution (Kirma et al. 2012). Abciximab was the most studied GP IIb/IIIa inhibitor for intracoronary use with conflicting evidence from different trials. One study showed intracoronary abciximab significantly reduced 30-day mortality and targeted vessel revascularization (TVR) in STEMI patients undergoing primary PCI compared to intravenous administration (Iversen et al. 2011). Another study that compared intracoronary to intravenous abciximab in STEMI patients undergoing primary PCI showed a significant reduction in the infarct size and extent of microvascular obstruction in the intracoronary arm (Thiele et al. 2008). These findings were however refuted by the CICERO trial that showed no improvement in myocardial reperfusion as assessed by ST segment resolution with intracoronary abciximab in STEMI patients undergoing primary PCI (Gu et al. 2010). Finally a large multicenter randomized trial that compared intracoronary to intravenous abciximab in STEMI patients undergoing primary PCI showed no difference in all-cause mortality, MI, or congestive heart failure at 90 days (Thiele et al. 2012). A meta-analysis of four randomized trials that included over 1000 patients undergoing primary PCI for STEMI treated with IC or IV abciximab showed a significant reduction in overall mortality with IC abciximab but no reduction in MACE (Shimada et al. 2012). Hence even though it has been confirmed that intracoronary administration of GP IIb/IIIa inhibitors has more platelet receptor occupancy, there appears to be no significant clinical benefit with this strategy. Consequently IC abciximab received a class IIb recommendation for use in select patients with STEMI in the recent ACC/AHA STEMI guidelines (O'Gara et al. 2013). Important trials are summarized in Tables 2, 3, and 4 according to the agent and clinical scenario in which it was tested.

Adverse Events

The most obvious side effect of GP IIb/IIIa use is bleeding. There is increased major and minor bleeding with GP IIb/IIIa use, but due to the different definitions used in clinical trials (TIMI and GUSTO), it is difficult to estimate an exact percentage risk. Also in the older GP IIb/IIIa trials where excess bleeding was identified, the dose of heparin used was higher with a higher activated clotting time (ACT) target. Now the recommended therapeutic ACT range for patients receiving GP IIb/IIIa inhibitors

Table 3 Trials examining the use of eptifibatide in different clinical scenarios

	ESPRIT (2000)	IMPACT (Tcheng et al. 1995)	PURSUIT (1998b)	EARLY ACS (Giugliano et al. 2009)
Clinical scenario	Elective and urgent PCI	Elective PCI (placebo vs. 4 h vs. 12 h infusion)	NSTEMI	NSTEMI (early vs. delayed eptifibatide)
Number of patients	2064	150	10,948	9492
Timing of drug	Downstream	Downstream	Upstream	Upstream
Dose	180 mcg/kg bolus × 2 2 mcg/kg/min for 18–24 h	90 mcg/kg bolus 1 mcg/kg/min for 4 or 12 h	180 mcg/kg bolus 2 mcg/kg/min for 72 h	180 mcg/kg bolus × 2 2 mcg/kg/min for 12 h
Other anticoagulants or antiplatelets	Heparin, ASA, ticlopidine, or clopidogrel	Heparin, ASA	Heparin, ASA	Heparin or low molecular weight heparin, ASA, clopidogrel
Invasive treatment arm	100 % (stent unspecified type)	100 % (PTCA or directional atherectomy)	23 % (PTCA or BMS)	100 % (PCI or CABG)
Primary outcome	Death, MI, urgent TVR, or thrombotic bailout at 48 h	Death, MI, or urgent revascularization	Death or nonfatal MI at 30 days	Death, MI, recurrent ischemia needing urgent revascularization, or thrombotic bailout at 96 h
Results	10.5 vs. 6.6 % ($p = 0.0015$) Terminated early due to efficacy	4.1 %(12 h) vs. 9.6 % (4 h) vs. 12.2 % (placebo)	14.2 vs. 15.7 % ($p = 0.04$)	9.3 vs. 10% (p = ns)

PCI percutaneous coronary intervention, *NSTEMI* non-ST elevation myocardial infarction, *ASA* aspirin, *PTCA* percutaneous transluminal coronary angioplasty, *BMS* bare metal stent, *CABG* coronary artery bypass graft surgery, *MI* myocardial infarction, *TVR* target vessel revascularization, *ns* nonsignificant

Table 4 Trials examining the use of tirofiban in different clinical scenarios

	PRISM (1998a)	PRISM PLUS (1998c)	TARGET (Topol et al. 2001)	ADVANCE (Valgimigli et al. 2004)
Clinical scenario	Unstable angina (tirofiban vs. heparin)	ACS (tirofiban + heparin vs. heparin)	Elective or urgent PCI for the United States, NSTEMI (tirofiban vs. abciximab)	NSTEMI
Number of patients	3232	1915	4300	202
Timing of drug	Upstream	Upstream	Downstream	
Dose	0.6 mcg/kg bolus 0.15 mcg/kg/min for 48 h	0.6 mcg/kg bolus 0.15 mcg/kg/min	10 mcg/kg bolus 0.15 mcg/kg/min for 18–24 h	25 mcg/kg bolus 0.15 mcg/kg/min for 18–24 h
Other anticoagulants or antiplatelets	Heparin, ASA	Heparin, ASA	Heparin, ASA, and clopidogrel	Heparin, ASA, clopidogrel, or ticlopidine
Invasive treatment arm	21 % (PTCA or stent)	30 % (stent)	100 % (stent)	100 % (stent)
Primary outcome	Death, MI, or refractory ischemia at 48 h	Death, MI, or refractory ischemia within 7 days	Death, nonfatal MI, or urgent revascularization at 30 days	Death, nonfatal MI, urgent TVR, and thrombotic bailout within 180 days
Results	3.8 vs. 5.6 % ($p = 0.01$)	12.9 vs. 17.9 % ($p = 0.004$)	7.6 % (tirofiban) vs. 6.0 % (abciximab) $p = 0.038$	20 vs. 35 % ($p = 0.01$)

ACS acute coronary syndrome, *ASA* aspirin, *MI* myocardial infarction, *TVR* target vessel revascularization, *PTCA* percutaneous coronary transluminal angioplasty

is 200–250 s. The more contemporary trials performed with these new recommendations showed lower bleeding rates comparable to heparin alone. None of the GP IIb/IIIa trials showed increase in intracranial or fatal bleeding. The incidence of clinically significant thrombocytopenia is approximately 2 % with abciximab and 1 % with tirofiban and eptifibatide.

Role of GP IIb/IIIa Inhibitors in the Current Practice

Antiplatelet and antithrombotic therapy forms the cornerstone of ACS therapy. In the current practice with an early invasive approach and routine potent $P2Y_{12}$ inhibitors

(clopidogrel, prasugrel, and ticagrelor), one may question the need for additional IV antiplatelet therapy. It is important to remember that even though aspirin and $P2Y_{12}$ inhibitors block one pathway of platelet activation, platelets continue to activate via various other pathways. In fact, the most potent platelet activator thrombin remains unchecked in patients with ACS. Hence GP IIb/IIIa inhibitors, which block the final common pathway of platelet activation, make it a more potent antiplatelet agent. With increasing use of newer oral $P2Y_{12}$ inhibitors (prasugrel and ticagrelor), which have a shorter time to peak antiplatelet effect, one may question the need for GP IIb/IIIa inhibitors. However, it should be noted that in the landmark trials that resulted in the approval of these drugs (TRITON TIMI 38 and PLATO), there was a 44 % and 64 % use of GP IIb/IIIa inhibitor, respectively, and hence the degree to which GP IIb/IIIa inhibitors contributed to reduction of events in these patients is unclear. Until recently GP IIb/IIIa inhibitors were the only intravenous antiplatelet agent available to cardiologists, but now cangrelor which is an IV $P2Y_{12}$ inhibitor is an alternative option for achieving rapid inhibition of platelet activation. The CHAMPION group of trials examined the use of cangrelor in patients undergoing PCI for various reasons (chronic stable CAD and ACS). CHAMPION PCI trial tested IV cangrelor use as an upstream strategy in patients undergoing PCI, and CHAMPION PLATFORM tested it as a downstream therapy that was initiated at the time of PCI. Both these trials compared cangrelor to clopidogrel and showed no benefit with cangrelor in terms of death, nonfatal MI, or urgent revascularization within 48 h of PCI, but there was a small reduction in stent thrombosis events (Bhatt et al. 2009; Harrington et al. 2009). CHAMPION PHOENIX trial then compared cangrelor to clopidogrel in patients undergoing elective or emergent PCI, and the drug was initiated at the time of PCI. This trial showed a significant reduction in the composite end point of death, MI, urgent revascularization, or stent thrombosis (Bhatt et al. 2013). Cangrelor was just approved by the FDA but its clinical role in daily interventional practice remains to be seen. Platelet thrombin receptor antagonist vorapaxar was studied in combination with GP IIb/IIIa inhibitors in patients with NSTEMI on dual antiplatelet therapy undergoing planned PCI in the TRACER trial. It was demonstrated that the combination of vorapaxar and GP IIb/IIIa inhibitors did not confer any ischemic benefit but increased bleeding (GUSTO moderate to severe) (Cornel et al. 2015). Even with all the new potent $P2Y_{12}$ inhibitors and bivalirudin, there continues to be a role for use of GP IIb/IIIa inhibitors in a carefully selected patient population such as high-risk ACS patient not adequately preloaded on $P2Y_{12}$ inhibitors, patient with peri-PCI complications such as side branch occlusion, STEMI patients with large thrombus burden particularly if the $P2Y_{12}$ inhibitor was just administered, and patients undergoing ad hoc PCI for stable CAD not adequately pretreated.

Conclusion

Glycoprotein IIb/IIIa inhibitors form an important class of medication in patients undergoing PCI for acute coronary syndromes to reduce recurrent MI and procedural complications. Although they were largely studied in the pre-$P2Y_{12}$ receptor inhibitor and balloon angioplasty era, there is still a role for GP IIb/IIIa inhibitors in current cardiology practice. There is no doubt that these medications have ischemic benefit but the associated increased bleeding risk warrants a careful patient selection to achieve a net clinical benefit.

Take Home Messages

- Glycoprotein (GP) IIb/IIIa inhibitors form an important class of medication in patients undergoing PCI for acute coronary syndromes to reduce recurrent myocardial infarctinon and procedural complications.
- Oral thienopyridines have replaced intravenous inhibitors of GP IIb/IIIa inhibotrs in many situations.
- Ischemic event reduction has been offset by an increased frequency of bleeding events.

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Dipyridamole and PDE Inhibitors

Paolo Gresele, Stefania Momi, and Emanuela Falcinelli

Abstract

Inhibition of platelet aggregation can be achieved either by the blockade of membrane receptors or by interaction with intracellular signaling pathways. Cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5-monophosphate (cGMP) are two critical intracellular second messengers provided with strong inhibitory activity on fundamental platelet function. Phosphodiesterases (PDEs), by catalyzing the hydrolysis of cAMP and cGMP, limit the intracellular levels of cyclic nucleotides thus regulating platelet function. The inhibition of PDEs may therefore exert a strong platelet inhibitory effect. In mammalian tissues, 11 structurally related but functionally distinct PDE gene families (PDE1–PDE11) have been described. Platelets possess three PDE isoforms, PDE2, PDE3, and PDE5, with different selectivity for cAMP and cGMP. Several nonselective or isoenzyme-selective PDE inhibitors have been developed, and some of them have entered clinical use as antiplatelet agents. This review will focus on the effect of PDE2, PDE3, and PDE5 inhibitors on platelet function and on the evidence for an antithrombotic action of some of them and in particular of dipyridamole and cilostazol.

Introduction

Inhibition of platelet aggregation is the cornerstone of treatment for the prevention of recurrent ischemic cardiovascular events. Platelet inhibition can be achieved either by blockade of membrane receptors or by interaction with intracellular signaling pathways. While receptor antagonism may provide high specificity, the inhibition of platelet signal transduction may display broader effects suppressing platelet activation regardless of the initial stimulus. Inhibition of signaling can be obtained either by interfering with platelet-activating second messengers or by amplifying the action of physiologic regulator of platelet function, like endothelial-derived nitric oxide (NO) and prostacyclin (PGI_2) which act by activating adenylyl and guanylyl cyclases, respectively, thus increasing intraplatelet cyclic adenosine 3′,5′-monophosphate (cAMP) and cyclic guanosine 3′,5′-monophosphate (cGMP). cAMP and cGMP are two critical inhibitory intracellular second messengers regulating fundamental platelet processes. In fact, elevation of platelet cyclic nucleotides interferes with almost all known platelet activation signaling pathways and thus blocks cytoskeletal rearrangement, fibrinogen receptor activation, degranulation, and expression of pro-inflammatory mediators. Indeed, cAMP and cGMP activate protein kinases that phosphorylate specific substrates (i.e., Rap1, MLCK, VASP, etc.), thus interfering with receptor/G protein activation and phospholipase C, protein kinase C, and MAP kinase activation and blocking cytosolic Ca^{2+} elevation and the reorganization of cytoskeleton (Daniel et al. 2002).

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Phosphodiesterases and Platelets

PDEs by catalyzing the hydrolysis of cAMP and cGMP to inactive 5′AMP and 5′GMP limit intracellular levels of cyclic nucleotides and thus regulate the amplitude, duration, and compartmentation of cyclic nucleotide signaling (Bender and Beavo 2006).

To date, more than 60 different isoforms of PDE have been described in mammalian tissues, grouped into 11 broad families (PDE1–PDE11) based on differences in their structure (Fig. 1), kinetics, regulatory properties, and sensitivity to chemical inhibitors (Maurice, Nat Rev Drug Discov 13:290-314, 2014). Alternative splicing and transcription start sites also contribute to the multiplicity of the different isoforms, many of which possess species-specific tissue and/or cellular distribution. Current data suggest that individual isozymes modulate distinct regulatory pathways in cells.

Given that PDEs are associated with many different functions, targeting pathological conditions by modulating individual PDEs has been the object of intense study (Table 1).

Functionally, PDEs can be classified in terms of their affinity for and rates of degradation of cAMP and cGMP. PDE1, PDE2, and PDE3 hydrolyze both cAMP and cGMP, and PDE4 and PDE8 specifically hydrolyze cAMP, whereas PDE5 specifically hydrolyzes cGMP. Concerning affinity for cyclic nucleotides, PDE3 isoforms are high-affinity enzymes, with KD's <150 nM, while other families, such as PDE1 and PDE5, have affinities in the μM range. For many PDEs, affinity for one substrate is so much higher than for the other as to render them functionally monospecific. PDE3 has a relatively high affinity for both cAMP and cGMP but a much lower efficacy of hydrolysis for cGMP, behaving essentially as a pure cAMP PDEs; it has also been referred to as cGMP-inhibited cAMP PDE because cGMP may competitively inhibit AMP hydrolysis (Grant and Colman 1984). Thus, in platelets, agents that activate guanylyl cyclase potentiate the effects of activators of adenylyl cyclase (Maurice and Haslam 1990).

Platelets express three PDE isoenzymes: PDE2, PDE3, and PDE5. Hidaka and Asano (Hidaka and Asano 1976) were the first to resolve the PDE activity of platelets into three distinct peaks: The first prefers cGMP as a substrate, with a Km of about 1 μM and is selectively inhibited by PDE5 inhibitors. The second hydrolyzes equally well cAMP and cGMP (Haslam et al. 1999) and is selectively inhibited by PDE2 inhibitors. The third has a high affinity for both cAMP and cGMP but hydrolyzes cAMP much more rapidly than cGMP and is selectively inhibited by PDE3 inhibitors. Thus, in platelets, cAMP is hydrolyzed by PDE3 and PDE2, and cGMP is hydrolyzed by PDE5 and PDE2 (Fig. 2); these isozymes account for at least 90 % of platelet PDE activity. Moreover, high concentrations of cGMP stimulate PDE2 (Bender and Beavo 2006). PDE2 inhibitors have been used mainly as research tools, but ongoing studies investigate their effectiveness for memory impairment and prevention of endothelial permeability in inflammation (Bender and Beavo 2006). PDE3 comprises two subfamilies, PDE3A and PDE3B, showing distinct and overlapping tissue and subcellular distributions. In situ hybridization studies have shown that PDE3A is highly expressed in the cardiovascular system, including the myocardium, VSMCs, and megakaryocytes, while PDE3B mRNA is detected in adipocytes (Park et al. 2007). It has been shown that PDE3A is the

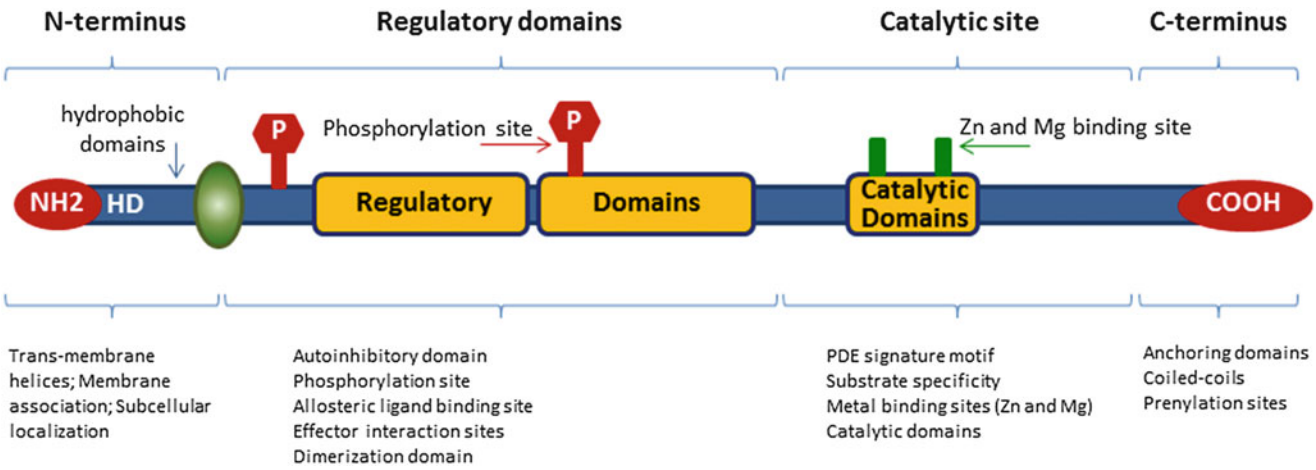

Fig. 1 General structure of PDE enzyme molecules

Table 1 Phosphodiesterase (PDE) families

Family	Substrate	Intracellular localization	Tissue expression	Specific inhibitors
PDE1	cGMP > cAMP	Cytosolic	Cardiac muscle, vascular smooth muscle, brain	Vinpocetine, IC86340, 8-MM-IBMX
PDE2	cGMP = cAMP	Membrane bound or cytosolic	*Platelets*, cardiac muscle, endothelial cells	EHNA, BAY 60-7550, PDP
PDE3	cAMP/cGMP	Membrane bound or cytosolic	*Platelets*, vascular smooth muscle	*Cilostazol*, milrinone, vesnarinone, lixazinone, anagrelide, olprinone, trequinsin, OPC-33540, dihydropyridazinone, siguazodan, SK&F 94120, CI 930, KCA-1490
PDE4	cAMP	Membrane bound or cytosolic	Cardiac muscle, vascular smooth muscle, brain, inflammatory and immune cells	Rolipram, etazolate, zardaverine
PDE5	cGMP	Cytosolic	*Platelets*, vascular smooth muscle	*Sildenafil*, E4021, DMPPO, CP461, MY-5445, NCX 911, DA-8159, zaprinast, *dipyridamole*, udenafil, lodenafil, avanafil
PDE6	cGMP > cAMP	Cytosolic	Retinal rods and cones	Sildenafil, zaprinast, dipyridamole
PDE7	cAMP>cGMP	Cytosolic	T-lymphocytes, B-lymphocyte, skeletal muscle, cardiac muscle	Dipyridamole
PDE8	cAMP	Membrane bound or cytosolic	Testis, eye, liver, kidney, skeletal muscle, embryo, ovary, brain	Zaprinast
PDE9	cGMP	Cytosolic or nuclear	Small intestinal smooth muscle, liver, kidney, lung, brain, testis, skeletal muscle, hearth, thymus	Methylxanthine, theophylline,
PDE10	cGMP > cAMP	Cytosolic or particulate	Testis and brain	None
PDE11	cAMP = cGMP	Cytosolic	Skeletal muscle, prostate, kidney, liver, pituitary and, salivary glands, testis	None

main subtype of PDE3 expressed in platelets (Sun et al. 2007).

PDE Inhibitors

Soon after the discovery of PDEs, it was found that caffeine is an effective inhibitor of PDE activity and a number of nonselective PDE inhibitors, including the caffeine analogue theophylline, entered clinical use. Later, several isoenzyme-selective PDE inhibitors have been developed as medicines or are currently under investigation for various disorders (Maurice et al. 2014).

This review will focus on the effects of PDE2, PDE3, and PDE5 inhibitors on platelet function and on their potential as antithrombotic agents (Table 2).

Dipyridamole

Dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidino-pyrimido-(5,4-d)-pyrimidine) was synthesized more than 50 years ago and initially used as a coronary vasodilator. However, it was soon shown that dipyridamole inhibited platelet aggregation (Born and Cross 1963; Elkeles et al. 1968) and thrombus formation in rabbits (Ahn et al. 1989), opening the way to the use of dipyridamole as an antithrombotic agent (Schwartz et al. 1988).

Mechanisms of Action

Dipyridamole affects platelet function by acting on different targets: it inhibits the reuptake of adenosine by red blood cells, in this way enhancing plasma levels of this vasodilator and platelet inhibitory nucleoside; it also acts as an inhibitor of PDE5 and PDE3, thus increasing intraplatelet cAMP and/or cGMP; it also acts as an antioxidant by scavenging free radicals that inactivate cyclooxygenase, thus enhancing PGI_2 biosynthesis.

Adenosine is released by tissues into the extracellular space as a breakdown product of ATP during ischemia, or by erythrocytes stressed by elevated shear forces, and is then taken up avidly by erythrocytes to keep plasma levels low. Inhibition of adenosine reuptake by dipyridamole is concentration dependent and reaches 90 % at 1 μM of dipyridamole

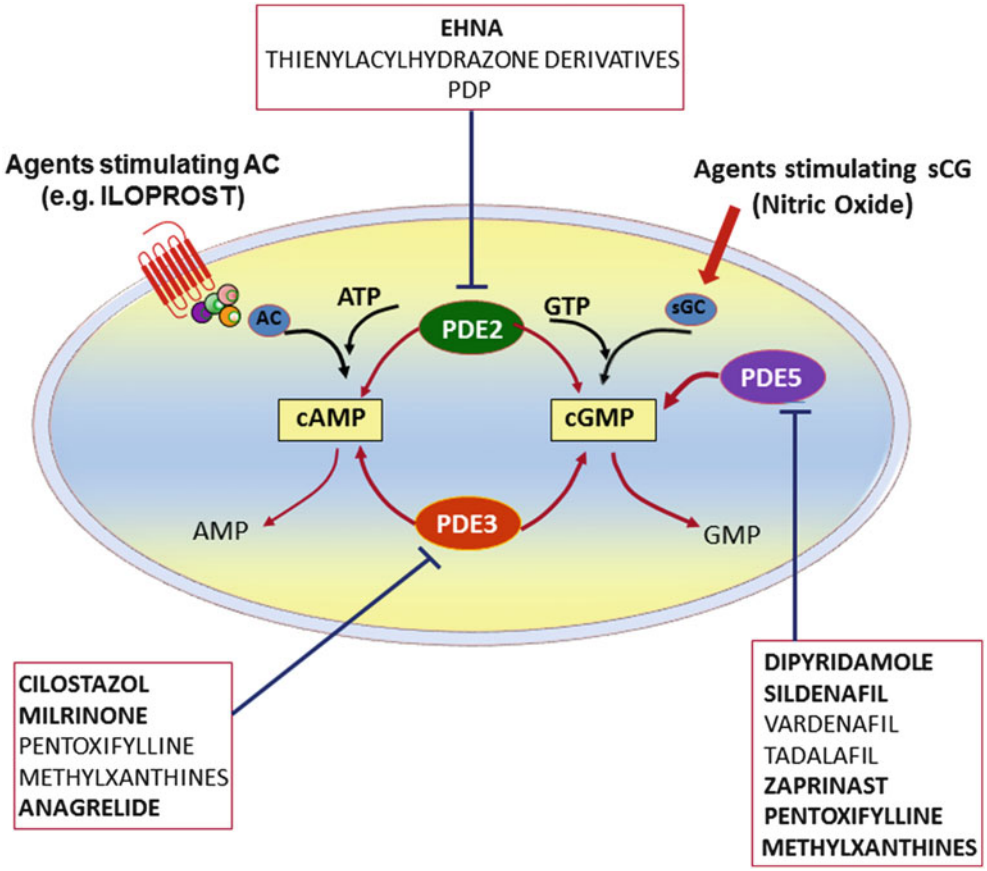

Fig. 2 Schematic representation of a platelet and the mechanisms regulating intraplatelet levels of cyclic nucleotides (cAMP and cGMP), the three isoforms of PDEs so far described in platelets, and their pharmacological inhibitors. In bold are the drugs discussed in the review

Table 2 PDE inhibitors and their clinical use

Family	Specific inhibitor	Use in clinical trial
PDE2	EHNA, BAY 60-7550, PDP, IC933, oxindole, ND7001, Aptosyn	No
PDE3	Cilostazol	Cerebrovascular disorders, peripheral vascular disease, intermittent claudication, prevention of stroke, peripheral arterial disease, diabetes mellitus
	Olprinone	Gastric acidosis, systemic inflammation after cardiopulmonary bypass
	Milrinone, cilostamide, amrinone, enoximone	Valvular disease, pulmonary hypertension, cardiac surgery
	Anagrelide	Essential thrombocythemia
	Vesnarinone, lixazinone	No
	Trequinsin, OPC-33540, dihydropyridazinone, siguazodan, SK&F 94120, CI 930, KCA-1490	No
PDE5	*Sildenafil*	Pulmonary hypertension, diabetes mellitus, endothelial dysfunction, ischemic stroke, schizophrenia, active digital ulcers, erectile dysfunction, gastroparesis, sickle cell anemia
	Tadalafil	Benign prostatic hyperplasia, erectile dysfunction, pulmonary hypertension, prostate cancer
	Vardenafil, lodenafil, avanafil	Erectile dysfunction
	Dipyridamole	Erectile dysfunction, ischemia-reperfusion injury, atherosclerosis, stroke, breast cancer, rheumatoid arthritis
	Udenafil	Erectile dysfunction, chronic obstructive pulmonary disease
	E4021, DMPPO, CP461, MY-5445, NCX 911, DA-8159, zaprinast	No

in whole blood (Klabunde 1993), a concentration in the range of those attained after oral administration to humans (0.5–6 μM). Dipyridamole inhibits platelet aggregation in whole blood, as assessed by impedance aggregometry, but not in PRP, both in vitro and ex vivo, by blocking the reuptake of adenosine (Gresele et al. 1983, 1986).

A stronger antiplatelet effect of dipyridamole in whole blood than in plasma was confirmed using different laboratory methods (De La Cruz et al. 1992; Perez Requeio et al. 1988; Muller et al. 1990; Saniabadi et al. 1991).

The modest PDE3-inhibitory action of dipyridamole increases the effects of adenosine and PGI_2, both stimulators of adenylyl cyclase, leading to inhibition of platelet activation (Soderback et al. 1991; Harker and Kadatz 1983).

Dipyridamole potentiates the inhibitory effects of NO on human and rabbit platelets (Sakuma et al. 1990) by inhibiting PDE5 and thus increasing vasodilator-stimulated phosphoprotein (VASP) production, an established marker of the NO/cGMP effects (Aktas et al. 2003), both in vitro and in vivo (Utz et al. 2001). However, the inhibition of PDE5 is detectable in vitro only at concentrations (100–200 μM) much higher than those attainable after oral administration.

The antioxidant properties of dipyridamole may contribute to its antithrombotic effect. In fact, not only dipyridamole enhances extracellular adenosine, which reduces superoxide anion generation by human neutrophils and directly scavenges oxygen and hydroxyl radicals, but it has direct antioxidant effects. In fact, dipyridamole has better antioxidant properties than ascorbic acid, α-tocopherol, and probucol (Iuliano et al. 1996; Pascual and Romay 1992).

The antioxidant activity of dipyridamole leads to the inhibition of leukotriene B4 production in vitro by stimulated white blood cells (Gresele et al. 1987). Moreover, based on its antioxidant properties, dipyridamole was shown to prolong prostacyclin production by "exhausted" vessel walls, preventing the autoinactivation of cyclooxygenase caused by enhanced peroxide formation (Deckmyn et al. 1984), an activity similar to the so-called physiological prostacyclin-regulating plasma factor (Deckmyn et al. 1985).

The redox state of dipyridamole regulates its antioxidant properties which appear to be mediated in vascular cells by suppression of NF-kB signaling (Chakrabarti and Freedman 2008).

Additional pharmacologic effects of dipyridamole that may be of relevance for prevention of atherothrombosis are the inhibition of vascular smooth muscle cell proliferation, the prevention of endothelium-leukocyte interactions (Kim and Liao 2008), the increase of antithrombotic properties of endothelial cells (Eisert 2001), the interference with leukocytes interaction with endothelium, the enhancement of IL-1-stimulated NO production by smooth muscle cells (Iimura et al. 1996), and the inhibition of inflammatory gene expression in platelet-monocyte aggregates (Weyrich et al. 2005). Very recently an additional interesting observation has shown that inhibition of multidrug resistance protein-4 (MRP-4)-mediated transport by dipyridamole increases aspirin entrapment in platelets and its in vitro effect on COX-1 activity (Mattiello et al. 2011), thus explaining in part the pharmacodynamic (Gresele et al. 1985) and therapeutic (Diener et al. 1996; Halkes et al. 2006) synergism with aspirin.

Antithrombotic Properties

The antithrombotic effects of dipyridamole have been evaluated in several animal models. Thrombus formation on air-injured carotid arteries of rabbits was reduced by dipyridamole, while salicylate increased it (Weber et al. 1990). In rabbits, the accumulation of radioactive fibrinogen at balloon angioplasty-treated carotid arteries was significantly reduced by dipyridamole (0.45 mg/kg/h for 4 h) as compared to heparin (Van Ryn et al. 2003).

More recently, in a model of chronic hind limb ischemia induced by the ligation of the common femoral artery branches in mice, dipyridamole (200 mg/kg b.i.d. for 7 days) was shown to enhance ischemia-induced arteriogenesis, thereby quickly restoring blood flow through a PKA-dependent NO pathway (Venkatesh et al. 2010).

Moreover, in a model of $FeCl_3$-induced femoral artery thrombosis in rats, the administration of dipyridamole (40 mg/kg), ineffective by itself, potentiated the antithrombotic effect of AICAR, an activator of the AMP-activated protein kinase (AMPK), suggesting that the combination of AMPK activators with PDE inhibitors could magnify their antiplatelet effect through a synergistic elevation of cGMP (Liu et al. 2013).

Clinical Studies

There is little clinical evidence that dipyridamole alone exerts an antithrombotic effect. Several studies instead have been carried out in combination with aspirin. Dipyridamole in combination with low-dose aspirin is associated with greater stroke risk reduction in patients with ischemic cerebrovascular disease. This was confirmed in two large clinical studies: the ESPS2 (Diener et al. 1996), in which 6602 patients with prior stroke or transient ischemic attack (TIA) were randomized to aspirin (50 mg daily), slow release dipyridamole (200 mg twice a day), the two agents in a combined formulation, or placebo, and the ESPRIT trial (Halkes et al. 2006), in which 2739 patients with previous TIA were randomized to aspirin (30–325 mg day) with or without dipyridamole (200 mg twice daily). A recent meta-analysis supports the higher efficacy of combination therapy

over aspirin alone (Verro et al. 2008). Based on these findings, the 2012 ACCP guidelines suggest dual therapy with extended-release dipyridamole plus aspirin (or clopidogrel) over aspirin monotherapy for stroke prevention after a first TIA or stroke (Lansberg et al. 2012). More recently, a meta-analysis including data from five clinical studies carried out in 4318 patients with previous TIA or stroke allocated to aspirin + dipyridamole and 4304 to aspirin alone concluded that the combination reduces the risk of stroke recurrence by 17 % with no rise of bleeding (Li et al. 2013). In addition, in patients with acute ischemic stroke or TIA, treatment with dipyridamole plus aspirin significantly lowered long-term mortality as compared with aspirin alone (Arnarsdottir et al. 2012). A large clinical trial, the PRoFESS study, was carried out to investigate whether aspirin 25 mg or extended-release dipyridamole 200 mg twice a day was not inferior to clopidogrel 75 mg once a day in 20,332 patients with previous stroke and did not meet predefined criteria for noninferiority but showed similar rates of recurrent stroke with the two treatments. Moreover, there were more hemorrhagic strokes with aspirin plus extended-release dipyridamole than with clopidogrel (Sacco et al. 2008). The degree of functional impairment (disability and cognitive decline) at 3 months after stroke was similar across treatment arms (Diener et al. 2008). The results of this large study are substantially inconclusive due to serious limitations in design, like the end point of noninferiority, the extremely stringent noninferiority margins, and the possible statistical fluke (Kent and Thaler 2008), and the considerable resources and effort that went into the study are unlikely to translate into any meaningful change in clinical practice (Anderson 2008).

Another condition for which there is evidence of efficacy of dipyridamole is secondary prophylaxis of thromboembolic events in patients with mechanical heart valves in combination with warfarin (Stein et al. 2001). Concerning the effects in other clinical indications, a recent overview of 27 randomized long-term secondary prevention trials involving 23,019 patients presenting with an arterial vascular disease (cardiac disease, cerebral ischemia, thrombosis of hemodialysis fistula, patients prone to develop atherosclerosis) comparing dipyridamole, alone or in combination with an antiplatelet drug other than dipyridamole (chiefly aspirin), with placebo found that there was no evidence that dipyridamole reduced the risk of vascular death, while it significantly reduced the risk of further vascular events (RR 0.88, 95 % CI 0.81–0.95). However, this benefit was statistically significant only in patients presenting after cerebral ischemia (De Schryver et al. 2006). Finally, among stented patients with high on-treatment platelet reactivity (HTPR), adding dipyridamole to clopidogrel does not

Table 3 Ongoing trials with cilostazol

Intervention	Condition	Primary end point	Estimated enrollment	Phase	ClinicalTrials.gov identifier
Cilostazol vs. placebo	Peripheral arterial disease, claudication	Difference in quality of life	30	Phase 4	NCT02374957
Cilostazol + simvastatin vs. cilostazol + pravastatin	Dyslipidemias, peripheral artery disease	AUC of simvastatin	20	Phase 1	NCT02431013
Cilostazol plus aspirin plus clopidogrel vs. aspirin or clopidogrel	Noncardioembolic cerebral infarction	Recurrence of symptomatic ischemic stroke	4000	Phase 3	NCT01995370
Probucol plus cilostazol vs. atorvastatin vs. atorvastatin plus probucol plus cilostazol	Severe hypercholesterolemia	Effect on mean Achilles tendon thickness	250	Phase 4	NCT02098460
Cilostazol vs. aspirin	Peripheral arterial disease	MACE—MALE	200	Phase 3	NCT02770274
Cilostazol vs. placebo	Coronary artery disease	Circulating EPC number	300	Phase 4	NCT02174939
Cilostazol vs. aspirin	Coronary artery disease	Severity of coronary artery stenosis	100	Phase 3	NCT02266030
Cilostazol vs. placebo	Mild cognitive impairment	Changes in MMSE	200	Phase 2	NCT02491268
Dual antiplatelet vs. triple antiplatelet	Acute myocardial infarction	MACCE	951	Phase 4	NCT01261832
Isosorbide mononitrate vs. cilostazol	Cerebral small vessel diseases, cognitive impairment stroke	Tolerability	60	Phase 2	NCT02481323
Cilostazol vs. placebo	Vasospastic angina	Chest pain frequency	100	Phase 3	NCT02087007
Ticagrelor or clopidogrel vs. cilostazol	Coronary artery disease	IPA	1350	Phase 4	NCT01955200
Triple antiplatelet therapy (TAT) vs. double-dose clopidogrel antiplatelet therapy (DDAT)	Coronary heart disease	TLF	3750	Phase 4	NCT01267734
Clopidogrel, ticagrelor, cilostazol	HTPR	MACE	70		NCT02101411

MACE major adverse cardiovascular events, *MALE* major adverse limb events, *MMSE* Mini-Mental State Examination, *MACCE* major adverse cardiovascular and cerebral events, *IPA* inhibition of platelet aggregation, *TLF* target lesion frequency, *HTPR* high on-treatment platelet reactivity

reduce platelet reactivity more than double-dose clopidogrel, and both strategies proved to be inadequate to overcome HTPR (Park et al. 2014).

Table 3 reports the ongoing trials with dipyridamole, alone or in combination with other antiplatelet drugs, registered in ClinicalTrials.gov.

Cilostazol

Cilostazol, a 2-oxo-quinoline derivative (6-(4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy)-3,4-dihydro-2(1H)-quinolinone), was registered in Japan and other Asiatic countries in 1988 and approved in the USA in 1999 for the treatment of intermittent claudication (Faxon et al. 2004).

Cilostazol is a specific and strong inhibitor of PDE3 in platelets ($IC_{50} = 0.2$ μM) and smooth muscle cells (SMC) where it diminishes intracellular calcium causing SMCs relaxation and inhibition of platelet activation (Shrör 2002). Cilostazol also inhibits adenosine uptake, thus enhancing adenosine levels that in turn enhance intracellular cAMP resulting in additional increases in cAMP (Shrör 2002).

Platelet Inhibition

Cilostazol inhibits both primary and secondary platelet aggregation induced by collagen, ADP, arachidonic acid, and epinephrine with IC_{50} values ranging from 3.6 to 15.0 μM, depending on the agonist (Kimura et al. 1985; Yasunaga and Mase 1985). It also suppresses the expression of P-selectin (IC_{50} 25 μM) (Kariyazono et al. 2001), TxB2 production, and PF4 and PDGF release and enhances the antiplatelet effects of PGI_2 (Igawa et al. 1990). Cilostazol inhibits shear stress-induced platelet activation in vitro, with an IC50 of 15.0 μM (Minami et al. 1997), and ex vivo not dissimilar to that exhibited by ticlopidine plus aspirin (Tanigawa et al. 2000). Among patients with acute myocardial infarction (AMI) undergoing coronary stenting, the association of cilostazol with clopidogrel plus aspirin resulted in a greater antiplatelet effect as compared with clopidogrel plus aspirin (Jeong et al. 2010). Moreover, the addition of cilostazol to clopidogrel gives greater inhibition of platelet aggregation than clopidogrel, either at the standard or at double dose, in clopidogrel low responders (Lee et al. 2010).

One potential benefit of the use of cilostazol over conventional antiplatelet therapy is the relatively short recovery time of platelet function (Iwamoto et al. 2003).

Cilostazol inhibits the expression of monocyte chemoattractant protein-1 (MCP-1), an initial trigger in the development of atherosclerosis and in human umbilical vein endothelial cells (HUVECs) by increasing intracellular cAMP (Nishio et al. 1997).

Cilostazol is rapidly absorbed and reaches peak plasma concentrations (775 ng/ml = 2.09 μM) at about 2.4 h after oral administration. In plasma cilostazol is largely bound to proteins (95–98 %), primarily albumin (Bramer et al. 1999; Suri et al. 1998). Specifically, metabolism of cilostazol occurs primarily via CYP3A5 and, to a lesser extent, CYP2C19, while <1 % of the administered dose is excreted unchanged in urine (Akiyama et al. 1985). CYP3A5 and CYP2C19 polymorphisms explain the substantial interindividual variability in the metabolism of cilostazol, with a coefficient of variation of about 40–60 % (Bramer et al. 1999; Yoo et al. 2010).

After oral administration, cilostazol and its metabolites, 3,4-dehydrocilostazol (OPC-13015) and 4-trans-hydroxy-cilostazol (OPC-13213), show a half-life of approximately 10 h, with a twofold accumulation during repeated administration (Shrör 2002).

Effect on the Bleeding Time

There is no evidence that cilostazol prolongs the bleeding time when compared to aspirin, clopidogrel, or ticlopidine (Tamai et al. 1999; Wilhite et al. 2003), even when used in combination with these antiplatelet drugs (Wilhite et al. 2003).

In a study in healthy men comparing ticlopidine, aspirin, and cilostazol, bleeding time was significantly prolonged by aspirin and ticlopidine, but not by cilostazol (Ikeda et al. 1987).

In patients with peripheral arterial disease (PAD), aspirin and clopidogrel alone significantly prolonged the bleeding time and even more when used in combination. In contrast, no prolongation of the bleeding time was observed with cilostazol alone, neither a further prolongation was seen when it was added to aspirin or clopidogrel (Comerota 2005).

Thus, unlike other antiplatelet drugs, cilostazol seems unique because patients receiving this drug do not show a significant prolongation of the bleeding time, but the reasons for this difference are still unknown. A recent in vitro study examining mural platelet thrombus growth on a collagen surface under high shear rate conditions in the absence of ADAMTS13 activity, i.e., mimicking blood flow in patients with thrombotic thrombocytopenic purpura, showed that cilostazol downregulates the size of mural platelet thrombi

without affecting their initial formation. This has been suggested as a reason why cilostazol does not significantly prolong bleeding time despite its strong antiplatelet activity (Yagi et al. 2012).

Animal Models

Cilostazol exerts antithrombotic activity in different animal models. Pulmonary thromboembolism induced by ADP and collagen in mice was reduced by cilostazol at 3 and 10 mg/kg per os, more effectively than by aspirin and pentoxifylline (Kimura et al. 1985). Cerebral infarction induced by the injection of arachidonic acid into the internal carotid artery of rabbits was reduced by 1 mg/kg cilostazol (Watanabe et al. 1986).

In a porcine model of totally occlusive thrombosis of the carotid artery induced by electrical injury, cilostazol significantly prolonged the time to occlusion and decreased thrombus weight more than ticlopidine (Kohda et al. 1999). In $FeCl_3$ venous thrombosis model in rats, oral administration of cilostazol 50 mg/kg significantly decreased venous thrombus weight (Kim et al. 2011).

Finally, the effects of clopidogrel, ticlopidine, and cilostazol on gastric bleeding induced by intraluminal perfusion of acidified ASA (25 mM aspirin acidified with 25 mM HCl) were studied in rats. Clopidogrel and ticlopidine (both 30 mg/kg), two $P2Y_{12}$ receptor inhibitors, increased gastric bleeding and ulcerogenic responses to acidified ASA, while cilostazol (30 mg/kg) suppressed them, making it a suitable candidate for combined use in dual antiplatelet therapy (Takeuchi et al. 2014).

Clinical Studies

Several studies have shown the superiority of cilostazol vs. placebo and also vs. pentoxifylline in increasing the walking distance (Thompson et al. 2002; Benedis et al. 2014) and quality of life of peripheral arterial disease (PAD) patients (Regensteiner et al. 2002).

Cilostazol was tested for secondary prevention of stroke in a randomized, placebo-controlled, double-blind trial in 1052 patients in Japan (Gotoh et al. 2000). Cilostazol reduced significantly the recurrence of ischemic stroke (−41.7 %, CI −9.2–62.5 %) as compared with placebo and the incidence of the combined end point MI, TIA, and intracranial hemorrhage. The benefits were achieved without a significant increase of bleeding (2.8 vs. 2.1 %) and with no fatal intracranial hemorrhages (Gotoh et al. 2000). These data seem to confirm a low bleeding risk with cilostazol. In the second Cilostazol Stroke Prevention Study (CSPS 2), in 2757 patients with a noncardioembolic cerebral infarction within the previous 26 weeks, cilostazol 100 mg twice daily was shown to be noninferior, and possibly superior, to aspirin 81 mg once daily, in preventing stroke recurrence (yearly rate of 2.76 %) ($n = 82$) in the cilostazol group and (3.71 %) ($n = 119$) in the aspirin group (HR 0.743, 95 % CI 0.564–0.981; $p = 0.0357$), and was associated with fewer hemorrhagic events (Shinohara et al. 2010). A meta-analysis of 12 double-bind, placebo-controlled trials in patients with atherothrombotic disease concluded that cilostazol is associated with a significant risk reduction of cerebrovascular events [relative risk (RR), 0.86; 95 % confidence intervals (CI), 0.74–0.99; $p = 0.038$] with no associated increase of bleeding (Uchiyama et al. 2009) and with no significant difference in the incidence of cardiac events (RR, 0.99; 95 % CI, 0.83–1.17; $p = 0.908$) (Uchiyama et al. 2009). Moreover, a more recent meta-analysis of nine studies (6328 patients) compared the efficacy and safety of cilostazol with other antiplatelet therapies in the prevention of recurrent cerebral ischemic events after an initial ischemic stroke. The study concluded that cilostazol, alone or in combination with aspirin, significantly reduced stroke recurrence (RR, 0.63; 95 % CI 0.52–0.76), poststroke intracranial hemorrhage (RR, 0.36; 95 % CI 0.21–0.63), and extracranial bleeding (RR, 0.62; 95 % CI 0.46–0.83) in patients with a prior ischemic stroke as compared with other antiplatelet therapies (Tan et al. 2015). A recent network meta-analysis of 24 randomized controlled trials for a total of 85,667 patients compared 11 antiplatelet therapies in patients with ischemic stroke or transient ischemic attacks treated for at least 1 year, concluding that long-term monotherapy was a better choice than long-term dual therapy and that cilostazol had the best risk-benefit profile for long-term secondary prevention after stroke or transient ischemic attack (Xie et al. 2015).

In a study in 1212 patients with acute coronary syndromes (ACS) undergoing percutaneous coronary intervention (PCI), cilostazol added to aspirin plus clopidogrel reduced the composite end point cardiac death, myocardial infarction, and stroke as compared to aspirin plus clopidogrel (Han et al. 2009). In agreement, a recent randomized trial in 514 patients undergoing percutaneous coronary intervention concluded that the addition of cilostazol to aspirin lowered the rates of cardiovascular and cerebrovascular events at 2 years compared to aspirin monotherapy (hazard ratio 0.61, 95 % CI 0.40–0.93, $p = 0.021$) (Ueda et al. 2016).

Recently, however, a multicenter randomized trial in 960 patients undergoing coronary drug-eluting stent implantation showed that the addition of cilostazol to aspirin and clopidogrel for 6 months did not reduce the composite adverse outcome of cardiac death, nonfatal myocardial infarction, ischemic stroke, or target lesion revascularization (Suh et al. 2011).

Table 4 Ongoing trial with dipyridamole

Intervention	Condition	Primary end-point	Estimated enrollment	Phase	Clinical trial.gov identifier
Dipyridamole vs. placebo	HIV infection	Changes in plasma levels of sCD14, sCD63, and IL-6	40	Phase 1 Phase 2	NCT02121756
Warfarin plus dipyridamole vs. aspirin plus dipyridamole on top of low molecular weight heparin	Cirrhosis, hypertension, splenectomy, venous thrombosis	PVT or spleno-mesenteric thrombosis	60	Phase 4	NCT02247414
Dipyridamole vs. placebo, dipyridamole + antihypertensives	Hypertension	Supine SBP	160	Phase 1 Phase 2	NCT00223717
Apixaban vs. any of aspirin, dipyridamole, carbasalate calcium, clopidogrel, no antithrombotic treatment	Cerebral hemorrhage, atrial fibrillation	Vascular death or nonfatal stroke	100	Phase 2	NCT02565693
Aspirin + dipyridamole + clopidogrel vs. DAPT	Stroke	Ordinal stroke severity at 90 days	4100	Phase 3	NCT01661322

SBP systolic blood pressure, *DAPT* dual antiplatelet therapy

Cilostazol has also been suggested to prevent post-stent restenosis (Weintraub et al. 2004). Cilostazol was compared with ticlopidine, both in addition to aspirin, in 397 patients undergoing elective coronary stenting for the rate of restenosis: restenosis tended to be lower with cilostazol, but not significantly (Ge et al. 2005). A recent study investigated whether periprocedural cilostazol affects the incidence of in-stent restenosis or target vessel revascularization after carotid artery stenting (CAS). The study group included 553 patients undergoing CAS, 207 of which (37.4 %) were treated with cilostazol, followed for 30 months. The incidence of ISR or TVR was significantly lower in the cilostazol-treated group than in the untreated group (1.4 vs. 6.4 %; log-rank $p = 0.006$) (Yamagami et al. 2010). A meta-analysis of five randomized controlled trials comparing triple therapy (cilostazol plus aspirin plus thienopyridines) with aspirin plus clopidogrel in 1597 patients undergoing coronary stenting showed that triple therapy reduced significantly more the 6-month restenosis rate (12.7 vs. 21.9 %; odds ratio 0.5; 95 % CI, 0.38–0.66; $p < 0.001$) (Jennings and Kalus 2010). A recent meta-analysis of 19 randomized trials involving 7464 patients undergoing coronary stent implantation showed that triple antiplatelet therapy (cilostazol plus aspirin plus clopidogrel) was associated with similar rates of death, nonfatal MI, ischemic stroke, and ST compared with dual therapy (aspirin plus clopidogrel), but with lower rates of target lesion revascularization (RR 0.67, 95 % CI 0.56–0.82, $p < 0.0001$) and target vessel revascularization (RR 0.65, 95 % CI 0.55–0.77, $p < 0.00001$), as well as less late loss of minimal lumen diameter (mean difference −0.14, 95 % CI −0.17 to −0.11, $p < 0.00001$) and less binary angiographic restenosis (RR 0.54, 95 % CI 0.45–0.65, $p < 0.00001$) (Chen et al. 2015). Both regimens had similar rates of bleeding, but triple therapy had significantly higher rates of headache, palpitations and tachycardia, rash, and gastrointestinal side effects, the most common side effects associated with cilostazol (Sorkin and Markham 1999). However, cilostazol-based triple antiplatelet therapy compared with dual therapy did not reduce major cardiovascular events and was associated with an increase in minor adverse events (Chen et al. 2015).

Finally, in a small study, cilostazol administration improved long-term patency after carotid artery stenting in 97 patients with high-grade carotid stenosis monitored for a 12-month period (0 vs. 15.7 %, $p = 0.03$) (Takigawa et al. 2010). In conclusion, several findings show promising effects of cilostazol, alone or even more in combination with other antiplatelet agents, but most studies are insufficiently sized, and further clinical research is warranted.

Table 4 reports the ongoing trials with cilostazol, alone or in combination with other antiplatelet drugs, registered in ClinicalTrials.gov.

Methylxanthines and Pentoxifylline

Naturally occurring methylxanthines were the earliest PDE inhibitors to be discovered, and among them caffeine (1,3,7-trimethylxanthine) was the first (Sutherland and Rall 1958). Some years later, theophylline (1,3-dimethylxanthine) and theobromine, more effective PDE inhibitors, were characterized (Butcher and Sutherland 1962). Ardlie and coworkers documented inhibition of platelet aggregation by caffeine and theophylline in 1967 (Ardlie et al. 1967). In a milestone study in 1971, Mills and Smith showed that adenosine increases cAMP in platelets and that methylxanthines prevent the conversion of cAMP to AMP, acting as PDE inhibitors, thus greatly increasing the inhibitory effects of adenosine on platelet aggregation (Mills and Smith 1971). Caffeine and theophylline act also as nonselective adenosine receptor antagonists (Evoniuk et al. 1987). In healthy

volunteers, 250 mg caffeine orally three times a day for 7 days reduced platelet aggregation, increased intracellular cAMP, and upregulated the platelet adenosine A_{2A} receptors (Varani et al. 1999). Recently, a 7-week pilot intervention study in healthy volunteers has shown that caffeine is not the only coffee constituent that contributes significantly to PDE inhibition in human platelet lysates and that other roasting-associated compounds, such as chlorogenic acid, polyphenols 5-CQA, and caffeic acid, play a role in the modulation of platelet cAMP (Montoya et al. 2014).

Pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-xanthine), a vasoactive drug used in patients with intermittent claudication (Jackson and Clagett 2001) and reported to reduce whole blood viscosity and to improve erythrocyte deformability, was shown to inhibit platelet aggregation in vitro in platelet-rich plasma (PRP) but at concentrations higher than those attainable in vivo (Nenci et al. 1981). However, given that the platelet inhibitory effect was potentiated by PGI2, it was suggested that the in vivo antiplatelet activity could be stronger (Weithmann 1980); moreover, pentoxifylline was found to inhibit platelet aggregation in whole blood more effectively than in PRP, due to the contribution of an adenosine uptake inhibitory effect on erythrocytes (Manrique and Manrique 1987). So far, there is no evidence that pentoxifylline reduces ischemic cardiovascular events (Jackson and Clagett 2001) neither that it enhances platelet inhibition in diabetic patients treated with dual antiplatelet therapy (Ueno et al. 2011).

Anagrelide

Anagrelide (Agrylin/Xagrid) (BL-4162A, 6,7-dichloro-1,5-dihydroimidazo[2, 1-6] quinazolin-2[3H]one monohydrochloride hydrate) is a potent inhibitor of PDE3 and a potent and broad spectrum inhibitor of platelet aggregation (IC_{50} = 36 nM) (Seiler et al. 1987).

During studies in humans, however, anagrelide was found to produce thrombocytopenia (Thiele et al. 2006); although the mechanisms through which anagrelide inhibits the megakaryocyte maturation are not completely understood, the drug has entered clinical use for patients with essential thrombocythemia (Silverstein et al. 1988).

Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), an inhibitor of adenosine deaminase (ADA), was shown to act as a selective PDE2 inhibitor with an IC50 of 3 μM and at least 50-fold selectivity over other PDEs (Dickinson et al. 1997). EHNA (20 μM) has no direct effect on platelet aggregation, but it potentiates the inhibition of thrombin-induced platelet aggregation by nitroprusside, a guanylyl cyclase stimulator (Dickinson et al. 1997). The use of EHNA as a tool to assess the role of PDE2 in platelets is limited by the low PDE2-inhibitory potency and by the concomitant adenosine deaminase inhibitory action.

Furthermore, a novel series of *thienylacylhydrazone derivatives* synthesized from natural safrole (4-allyl-1,2-methyldioxybenzene), a Brazilian natural product obtained from *Ocotea pretiosa*, have been studied as PDE2 inhibitors for their antiplatelet activity (Lima et al. 1999). The most potent of these inhibited platelet aggregation induced by arachidonic acid (IC50 0.2–3.1 μM) and collagen (IC50 0.9–3.4 μM) and effects enhanced by sodium nitroprusside without interference with ADP-induced aggregation, ATP release, and thromboxane (Tx)B2 production (Brito et al. 2010). Moreover, one of these induced a concentration-dependent relaxation of intact rat aortic rings (IC50=74 μM) (Silva et al. 2002).

Novel selective PDE2 inhibitors with nanomolar potency, such as 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6one (PDP), have recently been developed but not tested on platelets (Diebold et al. 2009).

Milrinone, a specific PDE3A inhibitor, inhibits arachidonic acid-induced platelet shape change and platelet aggregation induced by ADP, U46619, collagen, and calcium ionophore, both in whole blood and in platelet-rich plasma (Barradas et al. 1993), with IC50s as low as 1 μmol/l, a concentration lower than the concentration achievable after therapeutic administration (1.5 μmol/l), indicating that an antiaggregating effect milrinone can occur in vivo (Anfossi et al. 1996). Milrinone induces an elevation of intraplatelet cAMP in a dose-dependent way resulting in the inhibition of platelet aggregation (Manns et al. 2002).

Milrinone is currently in clinical use for congestive heart failure (Colucci 1991).

To date, only one family of PDE5, PDE5A, has been described. Three splice variants of PDE5A, PDE5A1/PDE5A2/PDE5A3, have been found in humans differing in their N-terminal region, but with similar Km for cGMP (Hidaka and Asano 1976).

Three *PDE5 inhibitors*, sildenafil (Viagra), vardenafil (Levitra), and tadalafil (Cialis), are currently in clinical use for erectile dysfunction.

Actually the first developed PDE5 inhibitor to be used in humans was zaprinast (M&B22948), originally designed as a mast cell-stabilizing drug for the treatment of allergic diseases (Murray 1993). The observation that cGMP elevation induced by zaprinast was associated with smooth muscle cell relaxation opened the possibility to use PDE5 inhibitors in cardiovascular diseases (Rudd et al. 1983). Zaprinast inhibits human platelet PDE5 with an IC_{50} of 0.3 μM and PDE2A with an IC_{50} of 42 μM. In association with SNP (1 μM), zaprinast led to a complete concentration-

dependent inhibition of serotonin release in washed platelets (IC_{50} ~ 1.6 μM) (Dunkern and Hatzelmann 2005). This compound was an unsuccessful clinical drug candidate, turned out to be weak and poor selective for PDE5. Consequently, a variety of compounds with key variations in the heterocyclic ring system of zaprinast were prepared leading to the identification in 1989 of sildenafil as a PDE5 inhibitor, which was 100 times as potent as zaprinast and highly specific in its action.

Sildenafil (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulfonyl]-4-methylpiperazine) is a potent PDE5 inhibitor, with high selectivity for human PDE5 over PDE2, PDE3, and PDE4 (>1000-fold) and moderate selectivity (>80-fold) over PDE1 (Schwartz and Kloner 2010). Sildenafil instead is only approximately tenfold more potent for PDE5 than for PDE6 (an enzyme found in the photoreceptors of the human retina), and this explains the frequent side effect of flashes of blue light with its use. Platelet PDE5 is inhibited by sildenafil with an IC_{50} of 6.3 nM (Wallis et al. 1999). While sildenafil alone had no effect on platelet function, it potentiated the inhibition by sodium nitroprusside of rabbit and human platelets (Wallis et al. 1999).

Sildenafil is rapidly absorbed after oral administration, with ~40 % bioavailability. Plasma concentrations peak within 30–120 min (median, 60 min), and the drug is primarily metabolized by hepatic cytochrome P450 3A4 (major route) and 2C9 (minor route) which convert it to an active N-desmethyl metabolite that possesses 50 % of the parent drug's potency for PDE5 (Cheitlin et al. 1999).

The administration of sildenafil (100 and 50 mg in healthy volunteers) inhibited collagen-induced aggregation (100 mg), with an additive effect when combined with nitrates (ISDN, 10 mg) (Berkels et al. 2001). Moreover, ADP-stimulated $\alpha_{IIb}\beta_3$ receptor activation was inhibited by sildenafil (100 mg) (Halcox et al. 2002). A study in healthy men showed that bleeding time was significantly prolonged (+72 %) 1 h after sildenafil intake (100 mg), with recovery after 4 h, whereas a lower dose (50 mg) did not alter the bleeding time (Berkels et al. 2001). Sildenafil was also reported to increase the incidence of epistaxis in patients on concomitant vitamin K antagonists and in combination with heparin had an additive effect on bleeding time in rabbits (Galie et al. 2010). Given the relatively low selectivity of sildenafil for PDE5, other more selective PDE5 inhibitors have been developed: vardenafil (Kd of 0.6–0.7 nM; selectivity over PDE6, approximately 16-fold) (Young 2002) and tadalafil (Kd range of 0.9–6.7 nM, 200–700 times more selective for PDE5 than PDE6) (Corbin and Francis 2002). In patients with erectile dysfunction associated with cardiovascular risk factors (dyslipidemia, hypertension, and smoke), tadalafil enhanced cGMP and significantly reduces P-selectin expression in platelets (De Bon et al. 2010).

Summary

Antiplatelet therapy is the mainstay of treatment of patients with acute cardiovascular ischemic events and of secondary prophylaxis of patients with a previous ischemic event. Despite great progress, even optimal antithrombotic therapy still does not offer satisfactory protection against cardiovascular events and is associated with serious risk of hemorrhagic side effects. Agents targeting thromboxane production or activity, ADP, and fibrinogen binding to platelets have proven of benefit but have also shown limitations.

An alternative, appealing strategy is to develop agents interfering with intracellular signaling pathways (Gresele et al. 2008). PDE inhibitors by increasing crucial intraplatelet second messengers, such as cGMP and cAMP, have great potential for platelet inhibition. In fact, differently from aspirin or clopidogrel, not only do they inhibit platelet activation induced by all stimuli, they also exert beneficial cardiovascular effects through their capacity to regulate the interaction of platelet with vascular smooth muscle cells in the setting of ischemic cardiovascular disease. However, the widespread distribution of PDE in the body renders it difficult that an effective antiplatelet action be achieved without significant unwanted effects. Moreover, the reversibility of the effect of most clinically available PDE inhibitors on their target may represent a serious limitation for their antithrombotic effectiveness in long-term secondary prophylaxis. It is now established that continuous platelet inhibition is required in order to obtain an effective antithrombotic protection and the reversibility of the blockade of the target with temporary restoration of platelet function in patients at high cardiovascular risk may be associated with enhanced ischemic events, as shown by the negative experience with orally active $\alpha_{IIb}\beta_3$ antagonists (Curtin and FitzGerald 2000). It is not a chance that antiplatelet agents currently used in secondary prevention of ischemic events, i.e., aspirin and thienopyridines, act both by irreversibly blocking their target. A deeper understanding of the physiology of PDEs in platelets and other tissues, the development of techniques allowing to target PDE inhibition to platelets, and the development of irreversible or long-acting isoenzyme-selective PDE inhibitors may potentially lead to advances in antiplatelet therapy.

Take Home Messages

- Continuous platelet inhibition is required to obtain an effective antithrombotic protection.

(continued)

- PDE inhibitors by increasing crucial intraplatelet second messengers, such as cGMP and cAMP, have great potential for platelet inhibition.
- PDE inhibition in platelets and the development of irreversible or long acting isoenzyme-selective PDE inhibitors may potentially lead to advances in antiplatelet therapy.

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PAR Antagonists

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Abstract

Thrombin is a potent platelet agonist that acts through protease-activated receptors (PARs) on platelet surface. PAR-1 is considered the main thrombin receptor in human platelets, while the roles of PAR-4 are not completely understood. Vorapaxar is the most widely studied PAR-1 antagonist and is the first in its class to be approved for clinical use, with indication for secondary prevention of recurrent ischemic events in patients with previous myocardial infarction or peripheral artery disease. In the chapter we will review the peculiar mechanism of action of PARs, their roles in hemostasis and thrombosis, and the pharmacology of vorapaxar. We will also review the main clinical data with special attention on the delicate balance between prevention of thrombosis and risk of bleeding.

Protease-Activated Receptors

Platelets have central role in maintaining hemostasis at sites of vascular injury and at the same time are responsible for thrombotic disease, especially in the context of ruptured atherosclerotic plaques or endothelial damage. Platelets are activated by multiple pathways, and their inner mechanism of amplification leads to growth of initial platelet aggregates (Jennings 2009).

The primary mechanism for in vivo activation at site of vascular damage, and thus key for hemostasis, is the adhesion to the extracellular collagen matrix, leading to platelet activation and the formation of an activated platelet monolayer (Jennings 2009). The interaction between collagen and platelets occurs through von Willebrand factor linked on collagen platelets and glycoprotein (GP) Ib/V/IX complex on platelets and the direct binding of the GP VI, the major platelet-collagen receptor, and collagen (Jennings 2009; Roberts et al. 2004). The collagen-induced platelet activation is thought to be central in maintaining normal hemostasis (Farndale et al. 2004).

Activated platelets release other factors leading to the amplification of platelet activation. In particular activated platelets secrete ADP, which is stored at high concentrations in dense granules, and contribute to the expansion of the platelet activation process (Jennings 2009). Similarly, release of thromboxane A2 from activated platelets contributes to the amplification of platelet-mediated thrombus formation. These amplification mechanisms are important in both normal hemostasis and pathologic thrombus formation. In addition to collagen, thrombin can trigger platelet activation. Thrombin is the most potent platelet activator known as it acts at very low, nanomolar concentrations (Jennings 2009). The interaction between thrombin and platelet in humans is mediated by the PAR-1 and the PAR-4 receptors (Coughlin 2000; Macfarlane et al. 2001). Both receptors can independently mediate thrombin signaling, and PAR-1 is activated at low concentration of thrombin, while PAR-4 becomes active at high concentration (Coughlin 2000). Thus, PAR-1 is thought to be the main thrombin receptor on human platelet. The roles of PAR-4 are not well understood. It may serve as a "backup" receptor in an important system or act in conditions with high thrombin concentrations (Coughlin 2000). Alternatively it could mediate responses to proteases other than

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thrombin or provide other unknown contributions to platelet function. Nonetheless both receptors appear to contribute to intracellular signaling in response to thrombin. In fact, PAR-4 is activated and shut off more slowly than PAR-1, and release of calcium signaling in response to thrombin in human platelets is given by the contribution of both receptors (Coughlin 2000). What roles PAR-4 plays with PAR-1 antagonist pharmacology strategies are also unknown and important to understand. Is concomitant PAR-4 blockade needed to achieve a full efficacy of antagonism of thrombin-mediated platelet activation? Is the efficacy and safety of PAR-1 antagonist modified by variability in PAR-4 activity?

PAR-1 is a seven-transmembrane-span G-protein-coupled receptor, which is activated through a unique mechanism (Fig. 1) (Zhang et al. 2012). In fact the interaction of PAR-1 with thrombin is not a typical "ligand-receptor" interaction. Instead, PAR-1 acts as a protease substrate for thrombin. In turn thrombin binds the receptor transiently and removes the N-terminus unmasking a tethered ligand (SFLLRN), prior to dissociating (Chen et al. 1994). The tethered ligand in turn activates the PAR-1. Unlike "typical" receptors that can go through many rounds of activation by diffusible agonists, each PAR undergoes a single activation cycle (Zhang et al. 2012). The identification of PAR antagonists has been challenging because compounds must compete with high local concentration of the tethered agonist. PARs are ubiquitous in the human body with a variety of functions in different tissues and have roles in cell proliferation and inflammation (Macfarlane et al. 2001). A detailed review of these functions is outside the scopes of this chapter. Intracellular signaling of PAR-1 occurs through G proteins $G_{\alpha q}$, $G_{\alpha 12/13}$, $G_{\alpha i}$, and $G_{\beta\gamma}$ that mediate intracellular response (Angiolillo et al. 2010) (Fig. 2).

Activated PAR-1 is rapidly desensitized by phosphorylation and arrestin binding, which uncouples the receptor from G proteins. PAR-1 is then internalized through endocytosis and degraded by lysosomes, without being recycled (Arora et al. 2007; Trejo et al. 1998; Zhang et al. 2012).

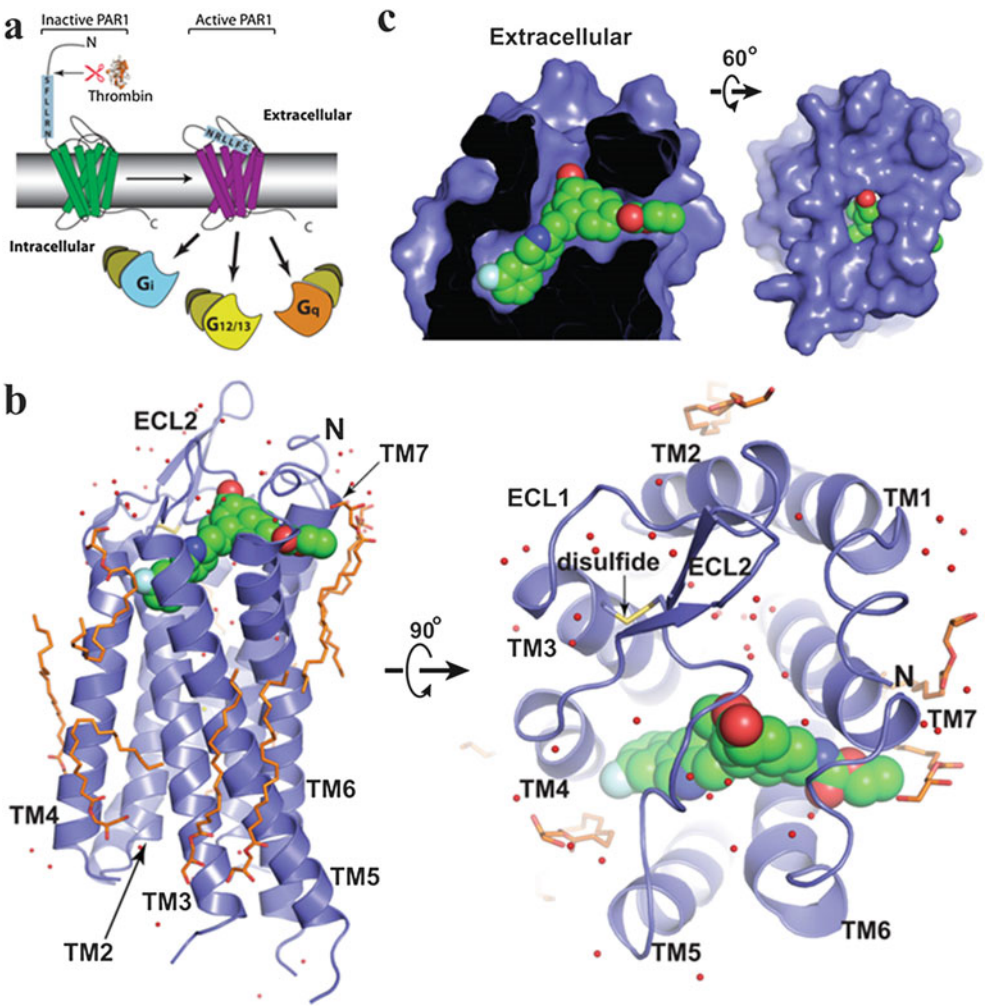

Fig. 1 PAR-1 activation and structure (From Zhang C, Nature. 2012 Dec 20; 492(7429): 387–392)

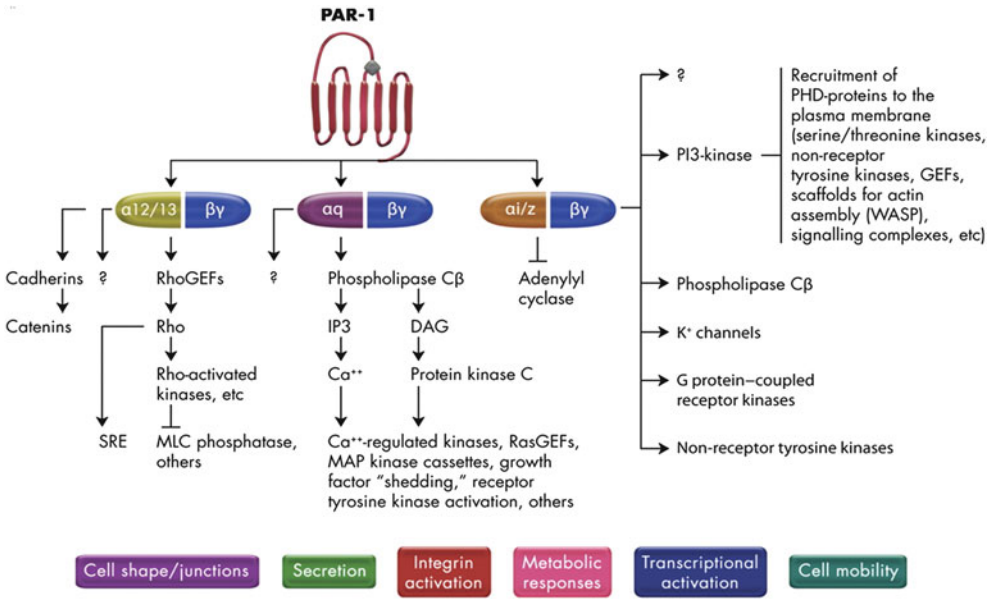

Fig. 2 Intracellular PAR-1 signaling pathway (From Angiolillo DJ, EHJ 2010)

Roles of PARs in Hemostasis

A lot of what we know about the roles of PARs in hemostasis comes from the work of Coughlin in mice (Cornelissen et al. 2010; Coughlin 2000). Mouse platelets express PAR-3 and PAR-4. PAR-3 is necessary for thrombin-mediated platelet activation at low thrombin concentrations; however, it is not associated with intracellular signaling but serves as cofactor to PAR-4. The thrombin signaling in mouse depends on PAR-4 (Coughlin 2005). The mouse dual receptor PAR-3/PAR-4 system provides a good dual receptor model like in humans, with PAR-3 resembling human PAR-1 given its high affinity for thrombin. PAR-3 -/- mice are a good model for human PAR-1 inhibition as higher thrombin concentration is needed to activate platelet (through PAR-4). PAR-4 -/- mice are totally unresponsive to thrombin and thus resemble concomitant PAR-1/PAR-4 inhibition in humans. The mouse models provide insights on what is the role of thrombin-mediated platelet activation in normal hemostasis. Complete thrombin deficiency is not compatible with life, and completely thrombin-deficient mice die during gestation. Fibrin (Fib -/-)-deficient mice show significant hemorrhagic tendency, but they can survive to old age. Combined fib -/- and PAR-4 -/- genetic makeup is also incompatible with life and mimics complete thrombin deficiency (Coughlin 2005). As expected, platelets of PAR-4 (-/-)-deficient mice are totally unresponsive to thrombin. However, they appear healthy and have no evidence of anemia or spontaneous bleeding. PAR-4 (-/-) female can support pregnancies, and platelets are normal in number and morphology (Coughlin 2005). In mice blood loss model PAR-3 (-/-) was not associated with increased bleeding time, unless concomitant $P2Y_{12}$ (-/-) genetic deficiency was present concomitantly (Coughlin 2005). A mild hemostatic defect can be detected from prolonged bleeding times only for high degree of injury (Hamilton et al. 2004). Similar results were observed with infusion of an intravenous PAR-1 antagonist SCH 530348 (analogue of vorapaxar), which did not cause increase in bleeding time in a cynomolgus monkey surgical blood loss model, unless a $P2Y_{12}$ inhibitor was administered concomitantly (Chintala et al. 2010). Overall these animal models support the concept that thrombin-mediated platelet activation is not critical to normal hemostasis. A clue on why thrombin-mediated platelet activation may be not as impactful on normal hemostasis comes again from PAR-4 -/- mice (Vandendries et al. 2007). A study with laser-induced thrombus formation and real-time digital fluorescence microscopy (Fig. 3) shows that compared to wild-type mouse, the PAR-4 (-/-) deficiency did not affect the formation of initial juxtamural thrombi at site of vascular injury, thus allowing hemostasis, but impaired the further propagation of platelet thrombus (Vandendries et al. 2007). So whenever the injury is small enough that significant growth of thrombus is not needed, PAR-mediated platelet activation may not be critical. In terms of therapeutic applications, blocking growth of thrombi without impairing the initial formation of a hemostatic plug may result in desirable effect, i.e., preventing formation of large occlusive thrombus, without losing hemostatic properties.

Even if impaired thrombin-mediated platelet activation does not significantly affect normal hemostasis in mice, it does appear to provide protection against thrombosis. PAR-4 (-/-) mice, whose platelet does not respond to thrombin, have been shown to have a reduced thrombotic response on a variety of arterial injury models (i.e., thromboplastin-induced pulmonary embolism, $FeCl_3$-induced thrombosis,

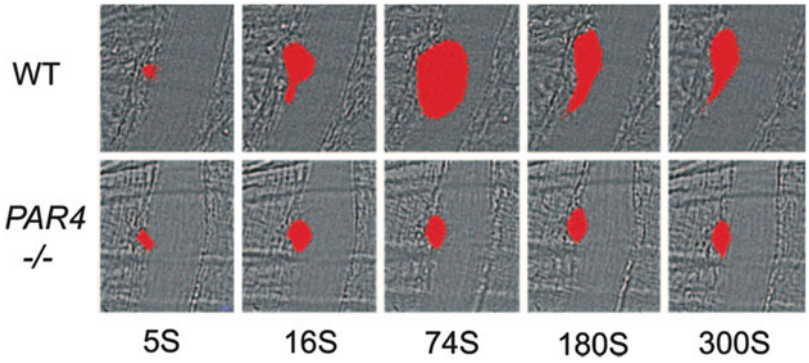

Fig. 3 Thrombus propagation in PAR-4 -/- mice (Vandendries ER, Proc Natl Acad Sci USA 2007)

laser injury-induced thrombosis of the cremasteric artery), supporting the hypothesis that blocking thrombin receptors on platelet may protect against thrombosis (Cornelissen et al. 2010). Data on protection against thrombosis derived from partial inhibition of thrombin-induced platelet activation were also derived from mice model. As described above PAR-3 (-/-) mice only respond to high concentration of thrombin, thus mimicking PAR-1 inhibition in human, where platelets only respond to high thrombin concentration through the PAR-4 receptor (Cornelissen et al. 2010). At low level of vascular injury (4 % $FeCl_3$), PAR-3 (-/-) mice were as protected from thrombosis as PAR-4 (-/-) mice and more protected than $P2Y_{12}$ (-/-) mice. At higher level of vascular injury (8 and 20 % $FeCl_3$), PAR-3 (-/-) mice did not show significant reduction in thrombus formation, unlike PAR-4 and PY12 (-/-) mice (Cornelissen et al. 2010). Taken together these results suggest that partial thrombin-mediated platelet activation (such as with PAR-1 antagonism) may be effective at low level of injury (which may correspond to spontaneous plaque rupture). At high level of arterial injury, such as during PCI, partial PAR blockade becomes less effective than complete PAR inactivation or $P2Y_{12}$ inhibition.

The totality of the animal model supported the hypothesis that blocking thrombin-mediated platelet activation can reduce thrombosis without impairing normal hemostasis.

Vorapaxar

Pharmacodynamics and Pharmacokinetics

Vorapaxar (Zontivity, Aralez) is a synthetic derived of himbacine, a compound found in barks of Australian magnolias (*Galbulimima baccata*) (Chackalamannil et al. 2005). Vorapaxar is an oral, potent, selective, competitive PAR-1 antagonist, with high affinity for the receptor (Chackalamannil et al. 2005). Vorapaxar does not interfere with other functions of thrombin, such as fibrinogen cleavage and fibrin generation, and the coagulation cascade is not affected. Vorapaxar is highly selective for the PAR-1 receptors (compared to others PARs) (Zhang et al. 2012). The binding pockets of vorapaxar on the receptor are very superficial, which is unusual for G-protein-coupled receptors (Zhang et al. 2012). There are three openings in the binding pocket that are narrower than vorapaxar. Thus, it remains unclear how the drug reaches the binding pocket, possibly through the membrane given the molecule is highly lipophilic (Zhang et al. 2012). The binding of vorapaxar with PAR-1 is reversible; however, after the drug attaches the binding pockets, those "close" resulting in a very slow off rate and a virtually nonreversible interaction (Zhang et al. 2012). Vorapaxar does not have significant on PAR-4-mediated response to thrombin (Judge et al. 2015).

Pharmacokinetics

Vorapaxar is rapidly absorbed following oral administration, and peak plasma concentration is achieved at 1–2 h after administration under fasting condition and a 45 min delay in peak concentration with a highly fat meal, but no changes in the area under the curve of concentration and a small change in peak concentration (https://www.merck.com/product/usa/pi_circulars/z/zontivity/zontivity_pi.pdf). The bioavailability of vorapaxar is nearly 100 %.

Vorapaxar is eliminated after liver metabolism mediated by CYP3A4 and CYP2J2 (Kosoglou et al. 2012). Coadministration with potent CYP3A4 inhibitors or inducers has been shown to increase (inhibitors) or decrease (inducers) exposure by approximately 50 %. The terminal elimination half-life is 8 days, resulting in a long persistent duration of platelet inhibition, and 50 % platelet inhibition is still seen after 4 weeks (https://www.merck.com/product/usa/pi_circulars/z/zontivity/zontivity_pi.pdf). Vorapaxar's metabolites are mainly eliminated through feces (58 %), with a smaller proportion of renal excretion (25 %). The major active circulating metabolite is the monohydroxy metabolite M20 (https://www.merck.com/product/usa/pi_circulars/z/zontivity/zontivity_pi.pdf). Vorapaxar and the

M20 are all virtually bound (≥99 %) to plasma proteins. Vorapaxar is highly lipophilic and the volume of distribution is >400 l.

Antiplatelet Effect

Vorapaxar inhibits ex vivo platelet aggregation induced by the thrombin receptor-activating peptide (TRAP), while it has no significant effect on ADP-induced platelet aggregation. In healthy volunteers, vorapaxar in 20 or 40 mg single loading doses promoted a >80 % inhibition of thrombin receptor-activating peptide (TRAP)-induced platelet aggregation within 1 h, and this effect was sustained for 72 h (Kosoglou et al. 2012). Multiple maintenance doses of 1 and 3 mg/day achieved nearly complete inhibition of 15 mcg TRAP-induced platelet aggregation at Day 7, while with 5 mg/day, complete inhibition was observed at Day 1. One interesting aspect of the antiplatelet effect of vorapaxar is that with increasing concentration, there is not a progressive decrease in platelet function. No antiplatelet effect is seen until the drug reaches a critical plasma concentration, and at that point an "on-switch" effect is seen, and >80 % platelet aggregation inhibition is reached (personal communication, Dr. John Strony). Studies on drug effect on platelet aggregation were also performed in the setting of the phase II among subjects undergoing PCI and in phase III (TRACER) among patients with non-ST-segment elevation acute coronary syndromes. In the phase II Thrombin Receptor Antagonist (TRA)-PCI trial, single loading dose of vorapaxar 40 mg yielded >80 % inhibition of TRAP (15 mcg)-induced platelet aggregation in almost 30 % of patients in 30 min, 70 % in 1 h, and 96 % in 2 h (Becker et al. 2009). At 2 h this effect was seen in 43 % and 53 % of patients in the vorapaxar 10 mg and 20 mg loading dose groups, respectively. At 30 days and 60 days, maintenance doses of 1 mg and 2.5 mg daily yielded >80 % platelet inhibition to TRAP in all patients. The TRACER Pharmacodynamic Substudy assessed the effect of vorapaxar at the doses used in the trial (40 mg loading, 2.5 mg maintenance dose), in NSTE-ACS patients largely (>90 %) treated with concomitant clopidogrel (Storey et al. 2014). Vorapaxar achieved a 97 % (94–98 % IQR) inhibition of maximum platelet aggregation to TRAP at 2 h after administration. This level of platelet inhibition was maintained with the 2.5 mg loading dose until the end of treatment. The study also suggested that platelet aggregation induced by TRAP in TRACER subjects at baseline (i.e., prior to vorapaxar administration) was lower compared to a group of healthy volunteers, suggesting that concomitant treatment with clopidogrel may have some effect on TRAP-induced platelet aggregation (Storey et al. 2014). At the same time, measurable decreases in ADP-induced platelet aggregation measured with VASP were seen with vorapaxar. These data, which need to be confirmed and may have been due to chance, suggest a possible interplay between $P2Y_{12}$ blockade and PAR-1 antagonism which may play a role in the antiplatelet effect when PAR-1 antagonists and $P2Y_{12}$ inhibitors are used concomitantly. A notable observation from the current study is that treatment with vorapaxar leads to reduced expression of PAR-1 after 1 month. The implication of this finding, i.e., a decreased sensitivity to thrombin-mediated platelet aggregation with consequent excessive inhibitory response to vorapaxar over time, needs to be further investigated.

Clinical Trials of Vorapaxar

Phase II Trials

The Thrombin Receptor Antagonist (TRA)-PCI trial assessed safety of vorapaxar which included patients undergoing elective coronary angiography with the intention to perform a PCI (Becker et al. 2009). Aspirin was given on the day of PCI, and patients were randomized to receive either a vorapaxar loading dose (10, 20, or 40 mg) or placebo before the coronary angiography. In cases of scheduled PCI, a loading dose of clopidogrel was given. Patients in the vorapaxar group who went ahead to receive a PCI were randomized in a blinded fashion to receive one of the three maintenance doses (0.5, 1.0, or 2.5 mg) for 60 days, and patients in the placebo group remained under treatment with placebo. Maintenance dose was not given for patients who did not undergo PCI, and those were followed in a separate cohort. A total of 1030 patients were enrolled: 573 underwent PCI and represented the primary cohort of the study, and 457 did not. The primary outcome, thrombolysis in myocardial infarction (TIMI) major or minor bleeding, occurred in similar rates among vorapaxar-treated and placebo-treated patients (3.3 % placebo vs. 2.8 % vorapaxar, OR 0.86, 95 % CI 0.30–2.47). However, there was a numerical trend toward higher bleeding rates with higher loading dose of vorapaxar (4.0 % with 40 mg). The rate of the composite outcome of death, major cardiac event, or stroke was numerically lower in the combined vorapaxar group, when compared with placebo (6 % in the combined vorapaxar group and 9 % in the placebo group, OR 0.67, 95 % CI 0.33–1.34). Myocardial infarction (MI) occurred in 4 % of the combined vorapaxar group and in 7 % of the placebo group.

Another phase II study with vorapaxar was conducted in Japan in patients with NSTE-ACS undergoing PCI (Goto et al. 2010). Participants were randomized to receive a vorapaxar 20 or 40 mg loading dose (followed by a 1.0 or 2.5 mg maintenance dose), or placebo, on top of standard care. The maintenance dose was given until completion of 60 days. PCI was performed in 92 patients, and the rates of TIMI major or minor bleeding were similar between the

pooled vorapaxar group and the placebo group (14 % in the pooled vorapaxar group and 10 % in the placebo group). Moreover, the occurrence of MIs was lower among vorapaxar-treated patients in comparison with placebo-treated patients (16.9 % in the vorapaxar group vs. 42.9 % in the placebo group; $p = 0.013$).

In trial with 90 Japanese patients with previous stroke, the use of vorapaxar (1.0 or 2.5 mg) on top of aspirin for 60 days did not increase the rates of adverse events in comparison with placebo (Shinohara et al. 2012). No TIMI major or minor bleeding was observed in vorapaxar-treated patients, whereas only one participant experienced a TIMI minor hemorrhage during study period. In addition, no MIs occurred.

The TRACER Trial

The Thrombin Receptor Antagonist for Clinical Event Reduction in Acute Coronary Syndrome (TRACER) trial is a phase III, double-blind, multicenter clinical trial which investigates vorapaxar versus placebo on top of standard antiplatelet therapy in preventing ischemic events in patients with NSTE-ACS (Table 1) (Tricoci et al. 2012). A total of 12,944 NSTE-ACS patients were included presenting with symptoms of ischemia within 24 h prior to hospitalization in addition to elevation of troponin/CKMB or high-risk electrocardiogram changes and at least one of the following: age greater or equal to 55 years, diabetes, peripheral arterial disease, previous MI, PCI, or coronary artery bypass graft (CABG). Key exclusion criteria were patients under the use of anticoagulants or inducers/inhibitors of CYP3A4 isoenzymes, history of bleeding diathesis, any previous intracranial bleeding, and severe valve heart disease. Participants were randomized to receive either vorapaxar, 40 mg loading dose at least 1 h before any revascularization procedure followed by 2.5 mg once-daily maintenance dose, or placebo. The duration of the treatment was for the entire duration of the study and a minimum of 1 year. Almost all of the participants received aspirin, and 91.8 % received clopidogrel. The median follow-up period was 502 days. Seven months after the completion of enrollment and 5 months before the planned conclusion of the follow-up, the study was terminated prematurely following a Data Safety Monitoring Board (DSMB). The DSMB, after that the minimum number of primary endpoint events were already achieved, detected an increase rate of intracranial hemorrhages (ICH) with vorapaxar and recommended interruption of the study.

The primary endpoint, composite of cardiovascular death, MI, stroke, rehospitalization for ischemia, or urgent coronary revascularization, occurred in a 2-year rate of 18.5 % in the vorapaxar group and 19.9 % in the placebo group (HR 0.92, 95 % CI 0.85–0.98; $p = 0.07$) (Table 2). Vorapaxar reduced the rate of the key secondary endpoint of cardiovascular death, MI, or stroke, in comparison with placebo (14.7 % vs. 16.4 % at 2 years, HR 0.89, 95 % CI 0.81–0.98; $p = 0.02$), mostly driven by a reduction in the

Table 1 Phase III clinical trials with vorapaxar

	TRACER	TRA 2P-TIMI 50
Year of publication	2011	2012
Number of patients	12944	26449
Patient population	High-risk NSTE-ACS	Previous MI: 67.3 % Previous stroke: 18 % PAD: 14 %
Arms	Vorapaxar 40 mg + 2.5 mg once daily Placebo	Vorapaxar 2.5 mg once daily Placebo
Background therapy	Aspirin: 99.1 % Clopidogrel: 91.8 %	Previous MI Aspirin: 98.1 % Thienopyridine: 78.1 % Previous stroke Aspirin: 81 % Thienopyridine: 23.6 % Dipyridamole: 19.4 % PAD Aspirin: 88 % Thienopyridine: 36.8 %
Primary efficacy endpoint	CV death, MI, stroke, recurrent ischemia with rehospitalization, or urgent coronary revascularization	CV death, MI, or stroke
Secondary efficacy endpoint	CV death, MI, or stroke	CV death, MI, stroke, or urgent coronary revascularization
Primary safety endpoint	Moderate or severe GUSTO bleeding	Moderate or severe GUSTO bleeding

Table 2 Clinical outcomes in the TRACER trial

TRACER endpoints	Vorapaxar event rate at 2 years (%)	Placebo event rate at 2 years (%)	HR (95 % CI); *p*-value
Primary efficacy endpoint	18.5	19.9	0.92 (0.85–1.01); 0.07
Secondary efficacy endpoint	14.7	16.4	0.89 (0.81–0.98); 0.02
MI	11.1	12.5	0.88 (0.79–0.98); 0.02
CV death	3.8	3.8	1.00 (0.83–1.22); 0.96
Stroke	1.9	2.1	0.93 (0.70–1.23); 0.61
Primary safety endpoint	7.2	5.2	1.35 (1.16–1.58); <0.001
TIMI clinically significant bleeding	20.2	14.6	1.43 (1.31–1.57); <0.001
Intracranial bleeding	1.1	0.2	3.39 (1.78–6.45); <0.001

rate of MI (11.1 % vs. 12.5 %, HR 0.88, 95 % CI 0.79–0.98; $p = 0.02$), especially spontaneous (type 1) MI. Because the primary endpoint did not meet statistical significance, the trial failed to prove superiority of vorapaxar, despite the nominal statistical significance of the triple endpoint.

Vorapaxar, added to standard of care with common use of dual antiplatelet therapy, increased the rate of Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) moderate or severe bleeding in comparison with placebo (7.2 % vorapaxar vs. 5.2 % placebo, 95 % CI 1.16–1.58; $p < 0.001$), as well as clinically significant bleeding by TIMI criteria (20.2 % vorapaxar vs. 14.6 % placebo, HR 1.43, 95 % CI 1.31–1.57; $p < 0.001$). The rates of intracranial bleeding and hemorrhagic stroke were significantly higher among patients in the vorapaxar group, as compared with placebo (1.1 % vs. 0.2 %, HR 3.39, 95 % CI 1.78–6.45, $p < 0.0001$; 0.3 % vs. 0.1 %, HR 2.73, 95 % CI 1.22–6.14, $p = 0.02$, respectively).

The results of TRACER suggest an overall modest benefit of vorapaxar when used largely in the setting of triple therapy with aspirin and thienopyridine. In fact even if the choice of the quintuple endpoint has likely caused the trial to be "neutral" and accepting a best-case scenario with an 11 % significant reduction of CV death, MI, or stroke, the benefit observed is largely offset by significant increase in bleeding. Nonetheless in our opinion the results of TRACER support the biological efficacy of PAR-1 blockade in ACS patients, as suggested by a reduction of the triple endpoint and the reduction observed in myocardial infarction, especially type 1. Interestingly no substantial effects of vorapaxar were seen in PCI-related MI. These results support the hypothesis, derived from mice studies and discussed before in this chapter, that PAR-1 antagonism may be protective when limited extension of vascular injury occur (i.e., spontaneous plaque rupture) and less with more extensive vascular damage (i.e., during coronary intervention). The significant increase in bleeding, including severe bleeding and ICH, should be interpreted in the context of triple therapy use, and it is similar to other trials where triple therapies were used, such as ATLAS-2 with rivaroxaban or APPRAISE-2 with apixaban (Alexander et al. 2011; Mega et al. 2012). Additionally, in TRACER, patients received a loading dose of 40 mg and concomitant use during the acute phase of parenteral antithrombin agents and about 20 % of patients concomitant GPIIb/IIIa inhibitors. Future studies are needed to establish whether vorapaxar can reduce ischemic events with an acceptable safety profile in ACS when used in the context of single or dual antiplatelet therapy, for example, by replacing aspirin.

The TRA 2P-TIMI 50 Trial

The Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA 2P-TIMI 50) trial compared vorapaxar versus placebo in addition to standard-of-care antiplatelet therapy in secondary prevention setting of patients with stable, chronic atherosclerotic disease (Table 1) (Dutta et al. 2012). A total of 26,449 participants were randomized which included three populations. The first, and largest (about 67 % of the population), included patients with recent myocardial infarction (2 weeks–12 months post-MI). The second (approximately 18 % of the population) was composed of patients with documented peripheral arterial disease (PAD). The remaining third included patients with recent stroke (2 weeks–12 months post-MI). Patients were randomized to receive 2.5 mg daily of vorapaxar, or placebo. No loading dose was used. Patients were treated concomitantly with aspirin and/or thienopyridine as per treating physician choice. Randomization was stratified by population and planned use of thienopyridine. After a median follow-up of 24 months, a high occurrence of intracranial bleeding events was observed with vorapaxar among patients in the vorapaxar group with previous stroke, including those who had a stroke during the study. The study treatment was interrupted in this group, while post-MI and PAD patients continued the trial to completion. The primary endpoint of the TRA 2P-TIMI 50 trial was revised after the completion of the TRACER trial and defined as the composite of cardiovascular death, MI, or stroke. This protocol modification was made before unblinding and trial termination.

Nearly all participants in the TRA 2P-TIMI 50 trial were treated with aspirin, 78 % of patients post-MI had concomitant thienopyridine, and 37 % of PAD patients had concomitant thienopyridine use.

At 3 years, vorapaxar significantly reduced the rate of the primary outcome in comparison with placebo (9.3 % in the vorapaxar group vs. 10.5 % in the placebo group, HR 0.87, 95 % CI 0.80–0.94; $p < 0.001$) (Table 3). The occurrence of the secondary outcome (composite of cardiovascular death, MI, stroke, or urgent coronary revascularization) was also reduced by vorapaxar, when compared with placebo (11.2 % in the vorapaxar group vs. 12.4 % in the placebo group, HR 0.88, 95 % CI 0.82–0.95; $p = 0.001$). Similar to TRACER, this result was mainly driven by a lower rate of MIs in the vorapaxar group (5.2 % vs. 6.1 %, HR 0.83, 95 % CI 0.74–0.93; $p = 0.001$ for vorapaxar vs. placebo). The rates of all-cause death (5.0 % in the vorapaxar group vs. 5.3 % in the placebo group; $p = 0.41$) and cardiovascular death (2.7 % in the vorapaxar group vs. 3.0 % in the placebo group; $p = 0.15$) were similar between study groups.

A significant increase in the rate of GUSTO moderate or severe bleeding events was observed with vorapaxar, in comparison with placebo (4.2 % in the vorapaxar group vs. 2.5 % in the placebo group, HR 1.66, 95 % CI 1.43–1.93; $p < 0.001$). Vorapaxar also increased the risk of clinically significant TIMI bleeding (15.8 % in the vorapaxar group vs. 11.1 % in the placebo group, HR 1.46, 95 % CI 1.36–1.57; $p < 0.001$) and intracranial bleeding (1.0 % in the vorapaxar group vs. 0.5 % in the placebo group, HR 1.94, 95 % CI 1.39–2.70; $p < 0.001$). The risk of ICH was mainly confined among those with prior stroke (2.4 % in the vorapaxar group vs. 0.9 % in the placebo group, HR 2.55, 95 % CI 1.52–4.28; $p < 0.001$). The excess seeing in ICH in those without stroke was more contained (0.6 % vs. 0.4 %, vorapaxar and placebo, respectively; $p = 0.049$). The rate of the net clinical outcome of cardiovascular death, MI, stroke, or GUSTO moderate or severe bleeding did not differ between study groups (11.7 % in the vorapaxar group vs. 12.1 % in the placebo group, HR 0.97, 95 % CI 0.90–1.04; $p = 0.40$).

The results of the TRA 2P-TIMI 50 trial demonstrated a benefit of vorapaxar in the secondary prevention setting in patients without history of stroke. While bleeding was also increased, the liability of vorapaxar on ICH was more limited in those without stroke. The population that appeared to have the highest benefit was the post-MI (Scirica et al. 2012). Among the 17,779 of 26,449 patients who were included based on a recent MI, the triple endpoint occurred at 3 years in 8.1 % with vorapaxar and 9.7 % with placebo (HR 0.80, 95 % CI 0.72–0.89; $p < 0.0001$). Moderate or severe bleeding were increased with vorapaxar (HR 1.61, 95 % CI 1.31–1.97; $p < 0.0001$), and a trend toward higher ICH rates was observed (0.6 vs. 0.4 %, $p = 0.076$). The benefit on CV death, MI, or stroke was more modest in the PAD population (HR 0.94, 95 % CI 0.78–1.14). No benefit was observed in the prior stroke population (HR 1.03, 95 % CI 0.85–1.25) (Scirica et al. 2012).

In 2014 vorapaxar received market approval in the USA by the Food and Drug Administration (FDA). A 2.5 mg once-daily regimen was approved for secondary prevention in patients with history of MI or with PAD (i.e., the TRA 2P population minus the stroke group). The drug is contraindicated in patients with previous stroke or transient ischemic attack due to increased risk of intracranial bleeding. Similarly, the drug has been approved in the European Union by the European Medicines Agency, for the post-MI indication. The difference was due to variation in procedural regulations between FDA and EMA in drug approval process.

The TRA 2P-TIMI 50 investigators have examined the efficacy and safety of vorapaxar in the trial population reflecting the FDA label (Magnani et al. 2015). A total of 20,170 patients randomized in TRA 2P-TIMI 50 trial met the current FDA label requirement (16,897 with a history of MI and 3273 with PAD). CV death, MI, or stroke at 3 years was significantly reduced with vorapaxar compared with placebo (7.9 vs. 9.5 %; HR, 0.80; 95 % CI 0.73–0.89; $p < 0.001$). GUSTO moderate or severe bleeding was higher in the vorapaxar group (3.7 vs. 2.4; HR, 1.55; 95 % CI 1.30–1.86; $p < 0.001$). ICH was 0.6 % with vorapaxar and 0.4 % with placebo, $p = 0.10$. For every 1000 patients treated for 3 years, vorapaxar prevented 16 CV death, MI, or stroke, at the expense of three more GUSTO severe bleeding and 13 GUSTO moderate or severe bleeding (Magnani et al. 2015).

Table 3 Clinical outcomes in the TRA 2P-TIMI 50 trial

TRA 2P-TIMI 50 endpoints	Vorapaxar event rate at 3 years (%)	Placebo event rate at 3 years (%)	HR (95 % CI); *p*-value
Primary efficacy endpoint	9.3	10.5	0.87 (0.80–0.94); <0.001
Secondary efficacy endpoint	11.2	12.4	0.88 (0.82–0.95); 0.001
MI	5.2	6.1	0.83 (0.74–0.93); 0.001
CV death	2.7	3.0	0.89 (0.76–1.04); 0.15
Stroke	2.8	2.8	0.97 (0.83–1.14); 0.73
Primary safety endpoint	4.2	2.5	1.66 (1.43–1.93); <0.001
TIMI clinically significant bleeding	15.8	11.1	1.46 (1.36–1.57); <0.001
Intracranial bleeding	1.0	0.5	1.94 (1.39–2.70); <0.001

Subgroup Analysis of Special Interest from TRACER and TRA 2P-TIMI 50

Secondary analyses from the two large phase III trials of vorapaxar have provided some very interesting insights on the effect of vorapaxar in certain subgroup, leading the way to possible future development on the drug.

Of great interest are the data from the TRA 2P-TIMI 50 on efficacy of vorapaxar or peripheral vascular outcomes. Clinical trials data supporting use of antiplatelet therapies in PAD are rather scant, and current guidelines recommend use of monotherapy with aspirin or clopidogrel (Rooke et al. 2011). Moreover PAD studies have generally focused on cardiac outcomes, rather than peripheral vascular/limb outcomes. In the TRA 2P-TIMI 50, peripheral vascular outcomes were adjudicated centrally by the clinical event committee. In an analysis among 3787 patients with PAD from the TRA 2P-TIMI 50, rates of hospitalization for acute limb ischemia were significantly reduced with vorapaxar (2.3 vs. 3.9 %; HR, 0.58; 95 % CI, 0.39–0.86; $p = 0.006$) (Bonaca et al. 2013). Also peripheral artery revascularization was significantly lower with vorapaxar (18.4 % vs. 22.2 %; HR, 0.84; 95 % CI, 0.73–0.97; $p = 0.017$). Numerical reduction was observed with vorapaxar on all indication for peripheral revascularization (acute limb ischemia, asymptomatic severe stenosis, chronic critical limb ischemia, claudication) and for all methods of revascularization (surgical or endovascular) (Gilchrist et al. 2015). These data suggest a possible role of vorapaxar in reducing peripheral vascular event and call for future trials comparing vorapaxar with standard of care (currently antiplatelet monotherapy) in the reduction of limb outcomes.

An interesting secondary analysis from the TRACER trial assessed the efficacy and safety of vorapaxar among patients who underwent coronary artery bypass surgery (CABG). In TRACER 1301 of 12,944 patients underwent CABG surgery during the index ACS hospitalization, and 1510 patients underwent CABG during the first 30 days after presentation (Whellan et al. 2014). The protocol recommended the continuation of the study drug before surgery and during the perioperative period. Among patients who underwent CABG, vorapaxar was associated with a 45 % reduction in the primary endpoint (HR, 0.55; 95 % CI, 0.36–0.83; $p = 0.005$), with a significant interaction ($p = 0.012$) between CABG and vorapaxar treatment. CABG-related TIMI major bleeding and chest tube drainage were not significantly different between vorapaxar and placebo. There was no increase in fatal bleeding, or need for reoperation with vorapaxar (Whellan et al. 2014). These results suggest that PAR-1 antagonism with vorapaxar could be an effective and safe strategy to prevent recurrent cardiovascular events following CABG surgery. The results of this study generate the hypothesis that vorapaxar may be effective in preventing ischemic events that are caused by graft failure. Future studies are required to prove this hypothesis and to establish if vorapaxar has a role in the treatment of patients undergoing CABG. One interesting aspect of the CABG analysis is the relatively good safety profile on CABG-related bleeding. It is possible that during CABG, when high concentrations of thrombin are generated, PAR-4-mediated platelet activation serves as effective backup mechanism in the presence of PAR-1 antagonist (Judge et al. 2015).

Both TRACER and TRA 2P-TIMI 50 have explored whether the efficacy and safety of vorapaxar were influenced by the concomitant treatment with thienopyridine (Bohula et al. 2015; Tricoci et al. 2014). Both studies came to the same conclusion that the concomitant treatment with thienopyridine does not affect the efficacy and safety of vorapaxar. In TRACER no significant interaction was found between concomitant long-term treatment with thienopyridine and the efficacy and safety of vorapaxar (Tricoci et al. 2014). Similarly in TRA 2P-TIMI 50, in which patients were stratified at randomization based on planned use of thienopyridine, vorapaxar reduced the CV death, MI, and stroke in patients who had planned thienopyridine use (HR 0.80, 95 % CI 0.70–0.91) and those who had no planned thienopyridine (HR, 0.75; 95 % CI 0.60–0.94) and negative interaction (interaction $p = 0.67$) (Bohula et al. 2015). GUSTO moderate or severe bleeding were increased with vorapaxar regardless of planned use of thienopyridine (interaction $p = 0.37$).

A total of 14,042 patients in the TRA 2P-TIMI 50 had a history of a coronary stent implantation before randomization, with 449 patients receiving a coronary stent during the trial (Bonaca et al. 2014). Vorapaxar was associated with lower rate of definite stent thrombosis (according to Academic Research Consortium definition) (1.1 vs. 1.4 %, HR 0.71, 95 % CI 0.51–0.98; $p = 0.037$). The majority (92 %) of stent thrombosis observed in the study occurred late or very late. In TRACER no significant reduction in ARC definite stent thrombosis was observed with vorapaxar (Tricoci et al. 2012). However, an angiographic core lab evaluation of stent thrombosis from TRACER suggested a low level of concordance in the adjudication of stent thrombosis between the adjudication committee (which based the adjudication mostly on cath lab reports) and an angiographic core lab (with independent, expert assessment of angiographies for the detection of stent thrombosis).

Atopaxar

Atopaxar is another potent PAR-1 antagonist, with a slower onset of action (3.5 h) than vorapaxar. It is metabolized by the liver (CYP3A4), with a shorter terminal half-life (23 h), and primarily fecal excretion.

Two randomized phase II trials were performed in Japan to assess safety of atopaxar in patients with coronary disease. A total of 241 participants with NSTE-ACS were included in the ACS trial (treated for 12 weeks), whereas 263 participants with high-risk stable coronary artery disease were included in the CAD trial (treated for 24 weeks). Patients were randomized to receive one of three dosing regimens of atopaxar (50, 100, or 200 mg), or placebo. In the ACS study, a 400 mg atopaxar loading dose or placebo was given after randomization. More than 90 % of the ACS patients and approximately 40 % of the CAD participants received dual antiplatelet therapy with aspirin and a thienopyridine. Overall, the rates of bleeding events defined by the Clopidogrel in Unstable angina to Prevent Recurrent Events (CURE) criteria were low. In the ACS study, only two major bleeding events occurred in the placebo arm, and one minor bleeding was seen in the atopaxar 200 mg group. In the CAD patients, one major bleeding occurred in the atopaxar 100 mg group, and one minor hemorrhage was observed in the atopaxar 200 mg arm. Numerically higher rates of TIMI-defined bleeding events were seen with atopaxar 200 mg, in comparison with placebo (23.0 % atopaxar 200 mg vs. 16.4 % placebo, $p = 0.398$, in the ACS study, and 13.2 % atopaxar 200 mg vs. 4.5 % placebo, $p = 0.08$, in the CAD study). No TIMI major hemorrhage was observed. The occurrence of major cardiac adverse events (MACE) was similar among atopaxar-treated and placebo-treated patients in the ACS study (5.0 % combined atopaxar group vs. 6.6 % placebo group, RR 0.76, 95 % CI 0.26–2.6; $p = 0.73$). In the CAD trial, the rate of MACE was numerically lower among atopaxar-treated patients (1.0 % in the combined atopaxar group vs. 4.5 % in the placebo group, RR 0.22, 95 % CI 0.04–1.11; $p = 0.07$).

The Lessons From Antagonizing the Cellular Effect of Thrombin-Acute Coronary Syndromes (LANCELOT-ACS) trial included 603 patients with NSTE-ACS within 72 h, to assess safety of atopaxar (O'Donoghue et al. 2011). Participants were randomized to receive atopaxar (all received a 400 mg loading dose and were assigned to receive 50, 100, or 200 mg as a maintenance dose) or placebo. The mean age was 62 years old, and 96 % were treated with aspirin, and 82 % with clopidogrel or ticlopidine. After 12 weeks of treatment, the occurrence of CURE-defined bleeding events was similar among study groups (3.1 % in the combined atopaxar group vs. 2.2 % in the placebo group; $p = 0.63$) (Table 4). The rate of CURE major bleeding was numerically higher in the atopaxar-treated patients (1.8 vs. 0 %; $p = 0.12$). No difference was observed in the rates of TIMI-defined hemorrhages between groups (9.2 % in the combined atopaxar group vs. 10.1 % in the placebo group, $p = 0.77$). The efficacy outcome (MACE) occurred in 8.0 % of the atopaxar-treated patients and in 7.8 % of the placebo-treated patients ($p = 0.93$).

The Lessons From Antagonizing the Cellular Effect of Thrombin-Coronary Artery Disease (LANCELOT-CAD) trial aimed to investigate the safety of atopaxar on a background of aspirin or dual antiplatelet therapy with aspirin and a thienopyridine for patients with high-risk stable CAD (Wiviott et al. 2011). A total of 720 participants were randomized to receive one of the three dosing regimens of atopaxar (50, 100, or 200 mg daily) or placebo for 24 weeks, with an additional follow-up period of 4 weeks. The primary endpoint was bleeding by CURE or TIMI criteria, and the secondary endpoint was the occurrence of MACE, both through week 24. The median age was 64 years, nearly all patients were using aspirin, and approximately 40 % were under dual antiplatelet therapy with aspirin and a thienopyridine. Atopaxar increased significantly the rates of bleeding by CURE criteria when compared with placebo (3.9 % in the combined atopaxar groups vs. 0.6 % in the placebo group, HR 6.82, 95 % CI 1.17–94.0; $p = 0.03$), in a dose-dependent fashion (Table 4). A numerically higher rate of hemorrhages by TIMI definition was also observed with vorapaxar, in comparison with placebo (10.3 % atopaxar vs. 6.8 % placebo, HR 1.52, 95 % CI 0.85–2.76; $p = 0.17$), mostly due to TIMI minimal bleeding. No difference was seen regarding the rates of MACE between study arms (2.6 % atopaxar vs. 4.6 % placebo, HR 0.57, 95 % CI 0.25–1.35; $p = 0.20$).

Transient elevation of hepatic enzymes and QTc interval prolongation were observed more frequently in the atopaxar groups when compared with placebo, especially with higher

Table 4 Clinical outcomes in the LANCELOT-ACS and LANCELOT-CAD trials

Endpoints	LANCELOT-ACS		LANCELOT-CAD	
Groups	Atopaxar combined ($N = 455$) (%)	Placebo ($N = 138$) (%)	Atopaxar combined ($N = 542$) (%)	Placebo ($N = 176$) (%)
Any CURE-defined bleeding	3.1	2.2	3.9	0.6
CURE-defined major bleeding	1.8	0	0.9	0
Any TIMI-defined bleeding	9.2	10.1	10.3	6.8
MACE	8.0	7.8	2.6	4.6

doses. There are no further studies ongoing or planned for atopaxar (source ClinicalTrials.gov).

PZ-128

As we have described earlier in this chapter, PAR-1 is a G-coupled receptor. The basic mechanism of activation of G-coupled receptors is based upon intracellular domains of receptors transmitting signaling to G protein. Therefore, intracellular domains of receptors may represent targets for drugs. Pepducins are synthetic compounds aimed at modulating the interactions between intracellular portion of the receptor and G protein (O'Callaghan et al. 2012).

Pepducins are made of a lipid component attached to a peptide that resembles one of the cytoplasmic loops or the C-terminal of the G-coupled receptor (O'Callaghan et al. 2012). The lipid component of the pepducin allows the molecule to cross the cell membrane and anchors it to cytosolic side (O'Callaghan et al. 2012). The peptidic component mediates the interactions with G proteins (O'Callaghan et al. 2012). The advantage of the pepducin technology is that libraries can be produced with different sequences in order to target the receptor of interest (O'Callaghan et al. 2012). Additionally, depending on the sequences, pepducin can act as agonist or antagonist (O'Callaghan et al. 2012). Different types of pepducins targeting a wide spectrum of receptors have been developed, including PAR-1, PAR-2, and PAR-4 (O'Callaghan et al. 2012). PZ-128 is a cell-penetrating lipopeptide pepducin that targets selectively the G protein associated with the PAR-1 on the inner side of the cellular membrane (Gurbel et al. 2015). Thus, ultimately PZ-128 uncouples the activated PAR-1 with intracellular signaling leading to inhibition of PAR-1-mediated platelet activation (Gurbel et al. 2015). PZ-128 is the first-in-class intracellular PAR-1 antagonist and is being developed as intravenous drug for prevention of acute thrombotic complications of PCI. PZ-128 was studied in a recent phase I study among patients with coronary artery disease or multiple coronary artery disease risk factors (Gurbel et al. 2015). The drug was administered as 1–2 h intravenous infusion, and inhibition of PAR-1-mediated platelet aggregation was studied using the agonist SFLLRN. The inhibitory effects of PZ-128 on platelet aggregation stimulated by SFLLRN were dose dependent with doses between 0.5 and 2 mg/kg. At 30 min 50–84 % inhibition of platelet aggregation was observed and was sustained to 6 h. At 24 h a 50 % recovery of platelet aggregation to SFLLRN was observed. Concomitant aspirin use greatly enhanced inhibition of platelet aggregation to SFLLRN, at the 0.5 mg/kg dose group. No significant effects on aggregation stimulated by a PAR-4 agonist, ADP, or collagen were seen, and no effects on bleeding time, PTT, international normalized ratio, and activated clotting time were observed. The plasma half-life of PZ-128 was 1.3–1.8 h, and no drug was detected in the urine. Given the fast onset of action and relatively short duration of action, PZ-128 represents an interesting intravenous antiplatelet agent, which warrants further investigation. A study of PZ-128 in subjects undergoing non-emergent PCI is currently planned (ClinicalTrials.gov NCT02561000).

PAR-4 Antagonists

Selective PAR-4 antagonists are being developed. The oral PAR-4 antagonist BMS-986141 (Bristol-Myers Squibb) will be investigated in a phase II trial in additional to aspirin for the prevention of brain infarction in patients with recent (<48 h) stroke or TIA (ClinicalTrials.gov NCT02671461). The study is currently planned to complete in mid-2018.

Conclusion

Thrombin is a known potent platelet activator, and inhibition of thrombin-mediated platelet activation thorough PAR-1 receptor antagonism is a novel mechanism to inhibit platelet aggregation and reduce thrombotic-mediated cardiovascular events. Data from large phase III clinical trials, in particular the TRA 2P-TIMI 50 trial, have confirmed that PAR-1 antagonisms with vorapaxar translate in the reduction of clinical events. This trial demonstrated a significant reduction in the rates of ischemic outcomes with vorapaxar in the secondary prevention among patients with recent MI or PAD, but without prior stroke or TIA, at the expense of an increase in major bleeding. The post-MI population appeared to benefit the most in terms of reduced cardiovascular outcomes, but intriguing effects on reduction in acute limb ischemia and need for peripheral revascularization were observed in patients with PAD treated with vorapaxar. The results of the TRA 2P study lead to the approval of vorapaxar for secondary prevention in patients with previous MI (USA and EU) or PAD (USA only) and without history of stroke or intracranial bleeding. Further research may address the role of vorapaxar in the setting of different antiplatelet strategies and different populations. Furthermore new PAR-1 antagonists are in development, and we will learn more about those drugs as new clinical studies are completed.

Take-Home Messages

Thrombin is the most potent platelet activator, and it acts through protease-activated receptors (PARs).

PAR-1 and PAR-4 are the thrombin receptors on human platelets, and PAR-1 is considered the main receptor because it is activated by low concentrations of thrombin, while the role of PAR-4 is not fully understood.

Animal studies suggested that impairment of PAR pathways did not result in significant bleeding tendency, thus supporting the hypothesis that PAR antagonism could result in antithrombotic effects and reduced bleeding liability.

Vorapaxar is an oral, highly selective, potent PAR-1 antagonist which significantly inhibits ex vivo platelet aggregation mediated to TRAP.

Vorapaxar was compared to placebo in two large phase III clinical trials, the TRACER trial in NSTE-ACS and the TRA 2P-TIMI 50, in secondary prevention among patients with recent MI, stroke, or PAD.

In both trials vorapaxar was studied in addition to standard of care, which largely included dual antiplatelet therapy with aspirin and clopidogrel.

The TRACER trial did not meet the primary endpoint, and a reduction in myocardial infarction was observed at the expense of increased risk in major bleeding and ICH.

The TRA 2P-TIMI 50 trial demonstrated that vorapaxar significantly reduced cardiovascular death, myocardial infarction, or stroke, in patients with prior MI or PAD and without history of stroke/TIA, at the expense of increased risk in major bleeding.

Vorapaxar (2.08 mg once daily equivalent to 2.5 mg vorapaxar sulfate) is approved for secondary prevention in patients with history of MI (USA and EU) or with PAD (USA only) and without history of stroke or TIA.

Atopaxar, another oral PAR-1, has been studied in phase II trials and has not been further developed.

Other PAR antagonists are currently in early-phase investigation, including the intravenous PAR-1 antagonist pepducin PZ-128 and the oral PAR-4 antagonist BMS-986141.

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Inhibitors of Platelet Adhesion to VWF and Collagen

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Abstract

Platelets play an essential role in thrombotic and hemostatic processes. Upon injury, platelets first adhere to the exposed extracellular matrix, predominantly via interactions with collagen and von Willebrand factor (VWF), resulting in hemostatic plug formation. Uncontrolled thrombus formation however results in occlusive complications leading to, e.g., myocardial infarction and ischemic stroke. New and more targeted antiplatelet therapies are essential to prevent such thrombotic complications. In this chapter, we describe different compounds that inhibit initial platelet adhesion. These include compounds that interfere with binding of VWF to collagen or platelet glycoprotein (GP) Ib and molecules that prevent platelet adhesion to collagen via blockade of either GPVI or integrin $\alpha_2\beta_1$. Results from the different preclinical animal and clinical patient studies demonstrate that inhibition of these interactions holds great promise as an antithrombotic strategy without significant increase of the bleeding risk.

Introduction

Vascular injury recruits platelets toward the exposed subendothelial extracellular matrix, of which collagen is the main thrombogenic component. Stable platelet adhesion to the extracellular matrix occurs in the order of tethering, rolling, activation, and firm adhesion and is depending on different platelet receptors. After immobilization of plasma VWF to exposed collagen fibers via the collagen-binding sites in the VWF A1 and A3 domains (Lankhof et al. 1996; Hoylaerts et al. 1997), high shear forces expose the cryptic platelet-binding epitope in the VWF A1 domain (structure of VWF illustrated in Fig. 1). As a result, VWF captures platelets via interaction of the A1 domain and the platelet glycoprotein (GP) Ibα receptor (Siedlecki et al. 1996). This induces tethering and rolling of platelets over the damaged vessel wall, but is not sufficient for stable platelet adhesion. Interaction with VWF brings platelets into close contact with exposed collagen allowing firm adhesion and subsequent activation by collagen via the two major platelet collagen receptors GPVI and integrin $\alpha_2\beta_1$. Thrombus formation is further propagated via α- and dense granule release, activation of integrin $\alpha_{IIb}\beta_3$, and facilitating a procoagulant surface for activation of the coagulation cascade. In healthy individuals, this controlled process results in the repair of the vascular injury, with maintenance of the normal blood flow through the vessel. However, uncontrolled thrombus formation might result in complete occlusion of the vessel, resulting in downstream tissue becoming ischemic. Myocardial infarction and ischemic strokes are examples of consequences of uncontrolled thrombus formation and are a major cause of death in the Western society. In this chapter, we focus on preclinical animal studies and clinical patient studies using inhibitors targeting the major platelet adhesion receptors GPIb, GPVI, and integrin $\alpha_2\beta_1$, as well as the main proteins involved in primary platelet adhesion, collagen and VWF.

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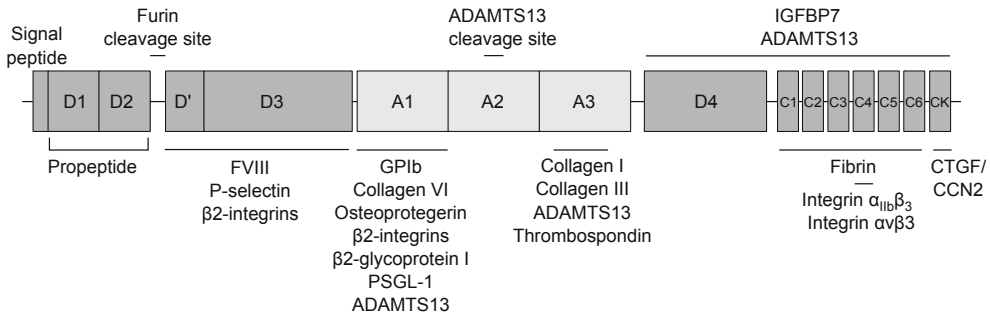

Fig. 1 Domain structure of a VWF monomer and the locations of interaction sites. *IGFBP7* insulin growth factor-binding protein-7, *CTGF*/*CCN2* connective tissue growth factor

The VWF–Collagen Interaction

VWF binds to collagen mainly via its A3 domain. Additionally, collagen, especially types IV and VI, can bind to the VWF A1 domain, which in addition is the domain responsible for platelet GPIb binding. A number of patients have been described with mutations in either the A3 or A1 domain that affect the binding to collagen (Flood et al. 2015). Interestingly, these mutations are not associated with a severe bleeding phenotype, suggesting that targeting this interaction might be relatively safe in terms of bleeding risk. Similarly, expression of a nonfunctional VWF A3 mutant in *Vwf*$^{-/-}$ mice resulted in delayed thrombus growth without affecting the bleeding time (Marx et al. 2008). Also in a mouse model of ischemic stroke, reconstitution of *Vwf*$^{-/-}$ mice with a VWF mutant defective in binding to collagen resulted in less ischemic stroke damage than in mice reconstituted with fully functional VWF, again without increase of bleeding (De Meyer et al. 2010). All these findings strongly suggest that interfering with VWF–collagen interaction might have a beneficial effect on thrombosis with little effect on bleeding. Hence, a number of inhibitory antibodies against the human VWF A3 domain have been tested in animal models.

Inhibitors of the Collagen–VWF Interaction that Bind to VWF

To overcome the problem that some inhibitory anti-VWF antibodies do not cross-react with murine VWF, Navarrete et al. expressed a mutant human VWF that can interact with murine GPIb in *Vwf*$^{-/-}$ mice (Navarrete et al. 2012). In this setup, inhibitory antihuman VWF A3 monoclonal antibodies (mAb), mAb505 and mAb203, significantly delayed vessel occlusion in arterioles, whereas no effect was seen in veins. This highlights the role of VWF A3 in arterial thrombosis and the antithrombotic potential of inhibiting the interaction between VWF A3 and collagen.

The antihuman VWF A3 mAb 82D6A3 was tested in a modified Folts model in baboons, where cyclic flow reductions due to thrombus formation were induced by mechanical injury followed by stenosis applied to the femoral artery (Wu et al. 2002b). An intravenous bolus injection did result in abolition of thrombus formation, without a marked effect on the bleeding time. The same antibody however did not have an effect on in-stent stenosis in baboons, pointing out that collagen–VWF binding has little impact on this process (De Meyer et al. 2007). 82D6A3 was humanized by variable domain resurfacing and grafting on the constant region of human IgG4, resulting in an antibody with similar in vitro affinities and activities as the murine one (Staelens et al. 2006). Also the antihuman VWF mAb SZ-123 was tested for its effects on cyclic flow reductions in the femoral artery of rhesus monkeys where injection of SZ-123 resulted in 100 % prevention of cyclic flow reductions without significant prolongation of bleeding time (Zhao et al. 2013). SZ-123 however is an antibody with dual action since in vitro and ex vivo experiments revealed that SZ-123 also indirectly inhibits ristocetin-induced VWF A1 binding to GPIbα.

Inhibitors of the Collagen–VWF Interaction that Bind to Collagen

Apart from compounds that inhibit VWF–collagen interaction by blocking VWF, also a number of collagen binders have been tested in vivo. In collagen III, a single high-affinity binding site for VWF was identified with RGQOGVMGF (O is hydroxyproline) as the minimal VWF-binding sequence (Lisman et al. 2006). So far, and somewhat surprisingly, inhibitory collagen binders were essentially isolated from hematophagous animals such as leeches, mosquitos, and blackflies. And as collagen binders, they usually have multiple effects, as most of them tend to inhibit both VWF and platelet integrin $\alpha_2\beta_1$ and/or GPVI binding. Simplagrin was derived from the salivary glands of the blackfly *Simulium nigrimanum* and binds to collagen (types I–VI) with high affinity (2–15 nM) (Chagas et al. 2014). In contrast to the other collagen binders, Simplagrin specifically inhibits VWF interaction with collagen type III and indeed binds to the RGQOGVMGF peptide with a similar affinity (11 nM) as to collagen. It furthermore

completely blocks platelet adhesion to collagen under flow conditions at high shear rates, without blocking GPVI or integrin $\alpha_2\beta_1$ interaction to collagen (Chagas et al. 2014). In mice, Simplagrin prevented laser-induced carotid thrombus formation without significant bleeding (Chagas et al. 2014).

Also aegyptin, a mosquito salivary gland protein, binds with high affinity (5 nM) to RGQOGVMGF and with lower affinity (μM range) to the peptides $(GPO)_{10}$ and GFOGER which, respectively, represent GPVI and $\alpha_2\beta_1$-binding sites in collagen (Calvo et al. 2010). Aegyptin prevents photochemically laser-induced carotid artery thrombus formation in the presence of Rose Bengal in rats, without significant bleeding, and protects mice from collagen- and epinephrine-induced (VWF-independent) thromboembolism (Mizurini et al. 2013).

The collagen-binding leech antiplatelet protein (LAPP) was isolated (Connolly et al. 1992) and cloned (Keller et al. 1992) from the leech *Haementeria officinalis* and inhibits both VWF and integrin $\alpha_2\beta_1$ binding to collagens I, III, and IV (van Zanten et al. 1995). However, LAPP had no effect in vivo on either collagen graft thrombosis in baboons (Schaffer et al. 1993) or on platelet deposition in an atherosclerotic rabbit model of angioplastic injury (Lyle et al. 1995).

Calin on the other hand was extracted from the saliva of the medicinal leech *Hirudo medicinalis* as a collagen binder (Munro et al. 1991). This compound also prevents both VWF and platelet $\alpha_2\beta_1$ binding to collagen (Harsfalvi et al. 1995; Depraetere et al. 1999) but, in contrast to LAPP, did have antithrombotic effects in vivo, as it prevented platelet deposition in the traumatized femoral vein of hamsters. No effects were seen on the skin bleeding time in the treated hamsters and also not when calin was applied topically in a standardized skin incision in hamsters or baboons (Deckmyn et al. 1995). By a direct comparison between LAPP and calin, it was suggested that the difference could maybe be ascribed to calin actually still consisting of two different inhibitors (Depraetere et al. 1999), and indeed later on saratin was isolated from the *H. medicinalis* saliva, cloned and expressed as a specific inhibitor of the VWF–collagen interaction (Barnes et al. 2001) although somewhat conflicting evidence was provided that saratin also is able to inhibit $\alpha_2\beta_1$ binding to collagen (White et al. 2007). Whatever the exact mechanism, topical application of saratin reduced platelet deposition and intimal hyperplasia in a rat carotid endarterectomy model (Cruz et al. 2001).

Interfering with the Interaction Between VWF and GPIb

As described above, the interaction between the VWF A1 domain and platelet GPIb is crucial for initial platelet adhesion at sites of vascular injury. As a result, the GPIb–VWF axis has become an attractive target for the development of new antithrombotic compounds. During the last decade, several agents that interfere with the GPIb–VWF interaction have been characterized, and some of these have already entered the first clinical trials.

Blocking the GPIb-Binding Site in the VWF A1 Domain

Back in 1997, Kageyama et al. prepared antihuman VWF antibodies in mice and selected the IgG AJvW2 with a conformational epitope in the VWF A1 domain (Kageyama et al. 1997). AJvW2 exerts a strong inhibitory effect on the interaction between VWF and GIPb as shown by impaired ex vivo VWF-mediated platelet adhesion and agglutination (Kageyama et al. 1997; Yamamoto et al. 1998). When used in canine and guinea pig models, AJvW2 abolished thrombus formation and showed a superior bleeding safety profile compared to antagonists of integrin $\alpha_{IIb}\beta_3$ (Kageyama et al. 1997, 2001). A humanized version of AJvW2 (AJVW200) was next generated, which also effectively inhibited thrombus formation in dogs, again with a safer bleeding profile than integrin $\alpha_{IIb}\beta_3$ inhibitors (Kageyama et al. 2002a, b). More recently, AJW200 was shown to reduce also venous thrombus formation and pulmonary thromboembolism in a rabbit model of venous thromboembolism (Takahashi et al. 2009). Moreover, in a rabbit embolic stroke model, administration of AJW200 with low-dose tPA was synergistic and resulted in significantly improved behavioral function following embolic stroke, showing the potential of VWF–GPIb inhibitors in thrombolytic therapy (Lapchack et al. 2013). In a first clinical study in 2003, AJW200 was well tolerated in human volunteers without bleeding complications (Machin et al. 2003), but no new clinical follow-up studies have been reported since then.

A second inhibitor that binds to the VWF A1 domain is GPG-290, a homodimeric recombinant fragment of human GPIbα. It is composed of the first 290 amino acids and conjugated to human IgG1Fc to form a homodimer. Two gain-of-function mutations (G233V/M239V) give GPG-290 an enhanced affinity for the VWF A1 domain. Because binding of GPG-290 to VWF inhibits the VWF–GPIb interaction, GPG-290 has been shown to exert antithrombotic activity in preclinical murine and canine models of arterial and venous thrombosis with a bleeding safety profile that was superior to clopidogrel (Hennan et al. 2006; Wadanoli et al. 2007; Brill et al. 2011).

Aptamers are nucleic acid macromolecules with a high affinity for a specific molecular target. The aptamer ARC1779 is a 40-nucleotide DNA/RNA aptamer that binds to the VWF A1 domain, thereby inhibiting the interaction between VWF and GPIb. The antithrombotic activity of

ARC1779 was demonstrated in several preclinical settings. ARC1779 reduced adhesion of platelets to collagen-coated matrices, formation of platelet thrombi on denuded porcine arteries, and formation of occlusive thrombi in cynomolgus monkeys (Diener et al. 2009). In a Phase I trial, ARC1779 was well tolerated in healthy volunteers. In a subsequent Phase II trial, ARC1779 effectively increased platelet counts in critically ill patients with thrombotic thrombocytopenic purpura by blocking spontaneous VWF-mediated platelet aggregation (Knobl et al. 2009; Jilma-Stohlawetz et al. 2011; Cataland et al. 2012). Since desmopressin aggravates thrombocytopenia in type 2B von Willebrand disease by the release of large and hyper-adhesive VWF multimers, Jilma et al. investigated whether ARC1779 can prevent desmopressin-induced thrombocytopenia in these patients, which was indeed the case (Jilma et al. 2010). Administration of ARC1779 led to reduced cerebral emboli signals in patients undergoing carotid endarterectomy (Markus et al. 2011).

Nanobodies are a novel class of therapeutic proteins, based on the smallest functional fragments of single-chain antibodies derived from the Camelidae family. They combine the advantages of conventional antibodies with important features of small-molecule drugs and are being developed for a range of diseases. ALX-0081 (later named caplacizumab) is a bivalent nanobody directed toward the A1 domain of VWF that specifically blocks the interaction of VWF with GPIb. ALX-0081 exerts potent activity in vitro and in vivo. Perfusion experiments with blood from patients with acute coronary syndrome demonstrated complete inhibition of platelet adhesion after addition of ALX-0081 (Ulrichts et al. 2011). In a modified Folts model in the baboon femoral artery, the in vivo efficacy and safety, reflected by the inhibition of thrombus formation and surgical bleeding, respectively, were assessed, and ALX-0081 showed a superior therapeutic window compared to clopidogrel and abciximab. The addition of a standard therapeutic combination of heparin, aspirin, and clopidogrel to ALX-0081 did not further enhance efficacy as compared to ALX-0081 monotherapy, but also did not increase surgical bleeding tendency (Ulrichts et al. 2011). Interestingly, ALX-0081 prevented thrombosis in the middle cerebral artery of guinea pigs and induced cerebral reperfusion when given immediately after or 15 min after complete occlusion, thereby reducing brain damage without inducing hemorrhage (Momi et al. 2013). In a Phase I single-center, double-blind, randomized, placebo-controlled study, the nanobody was well tolerated and safe in healthy volunteers (Bartunek et al. 2008). In a subsequent Phase I double-blind, randomized, placebo-controlled study, ALX-0081 was further evaluated in patients with stable angina undergoing PCI. In this study, multiple dosing of ALX-0081 resulted in sustained inhibition of VWF-mediated platelet aggregation without increasing the bleeding risk albeit that patients received standard therapy consisting of aspirin, clopidogrel, and heparin (Bartunek et al. 2010).

In the recent TITAN trial, a randomized, placebo-controlled, single-blind, parallel design study, caplacizumab improved standard of care of patients affected with acquired thrombotic thrombocytopenic purpura by a more rapid normalization of platelet numbers and lower occurrence of exacerbations with manageable side effects and bleeding episodes (Peyvandi et al. 2015). A Phase III "HERCULES" study has been initiated, which will further evaluate the efficacy and safety of caplacizumab in patients with acquired thrombotic thrombocytopenic purpura when administered in addition to the standard of care (ClinicalTrials.gov identifier: NCT02553317).

ADAMTS13: Reducing the Size and Activity of VWF

Besides blocking the platelet-binding site on VWF, another way to limit VWF activity is decreasing its size as indeed VWF activity is positively correlated with multimer size. Ultralarge VWF multimers are hyperactive and particularly thrombogenic and are released from endothelial cells and platelets upon stimulation with various secretagogues. To prevent spontaneous thrombosis, ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 repeats, member 13) cleaves the Y1605–M1606 bond in the VWF A2 domain, resulting in digestion of ultralarge VWF into smaller, less reactive molecules. Somewhat unexpected, also the carboxyl terminal domains of ADAMTS13 on their own, in the absence of the proteolytic metalloproteinase domain, were shown to directly inhibit thrombus formation (Bao et al. 2014). A deficiency of ADAMTS13 is associated with the devastating disease thrombotic thrombocytopenic purpura caused by VWF-mediated microvascular thrombosis. The antithrombotic potential of ADAMTS13 was demonstrated in various settings. Chauhan et al. showed that ADAMTS13 downregulates both platelet adhesion to exposed subendothelium and thrombus formation in injured murine arterioles and venules, demonstrating the natural antithrombotic activity of ADAMTS13 (Chauhan et al. 2006). The antithrombotic role of ADAMTS13 in venous thrombosis was recently confirmed as mice deficient for ADAMTS13 formed larger venous thrombi than wild-type mice (Tashima et al. 2015). Recombinant ADAMTS13 is being developed as a new therapeutic agent and has been shown to exert a protective role in mouse models of ischemic stroke (Zhao et al. 2009; Fujioka et al. 2010) and myocardial infarction (De Meyer et al. 2012; Gandhi et al. 2012).

Blocking the VWF-Binding Site on GPIbα

A recombinant fragment of VWF, VCL, was made with the intention to compete with VWF for binding to GPIb, and thereby decrease thrombus formation. VLC consists of VWF Leu-504–Lys-728, with a single intrachain disulfide bond linking residues Cys-509–Cys-695 (Gralnick et al. 1992). In an electrical injury-induced thrombosis model in dogs, it was demonstrated that VLC increased time to occlusion in coronary arteries (Yao et al. 1994). In a baboon model, VLC effectively abolished cyclic flow variations in stenosed, endothelium-injured coronary arteries (McGhie et al. 1994).

Our group generated and characterized the antihuman GPIb mAb 6B4 (Cauwenberghs et al. 2001), which has an epitope in GPIbα that consists of two sets of peptide sequences aligning within Lys-230–Val-242 and Tyr-259–Gly-262 resulting in an efficient inhibition of the VWF–GPIb interaction (Cauwenberghs et al. 2001). When injected into baboons, intact 6B4 IgG and its F(ab′)2 fragments caused almost immediate thrombocytopenia, whereas injection of the Fab fragments alone did not. Baboons pretreated with 6B4 Fab fragments showed significantly reduced platelet deposition onto the collagen-rich bovine pericardium (Cauwenberghs et al. 2000). These findings were extended by investigating the efficacy of 6B4 Fab fragments in high shear conditions using a modified Folts model in baboons, in comparison with an integrin $\alpha_{IIb}\beta_3$ antagonist (Wu et al. 2002a). Administration of lower doses of 6B4 Fab significantly reduced the cyclic flow reductions, whereas higher doses completely abolished the cyclic flow reductions without prolongation of the bleeding time. In this study, an anti-$\alpha_{IIb}\beta_3$ antibody also inhibited occlusive thrombus formation but induced a significant prolongation of the bleeding time. Later, a fully recombinant and humanized version of 6B4 Fab fragment was generated and characterized (Fontayne et al. 2006) and maintained its inhibitory capacities in vitro and in vivo in baboons (Fontayne et al. 2008).

Additionally, anfibatide, purified from the venom of the snake *Agkistrodon acutus*, was found to be able to bind GPIb, thereby inhibiting VWF binding. Anfibatide prevented thrombosis and vessel occlusion in murine laser-injured cremaster arteries and $FeCl_3$-injured mesenteric arteries (Lei et al. 2014). Moreover, in a murine model of ischemic stroke, anfibatide had a protective effect on cerebral ischemia and reperfusion injury (Li et al. 2015), confirming earlier studies that highlight the importance of the VWF–GPIb interaction in ischemic stroke (Kleinschnitz et al. 2007; De Meyer et al. 2010; Verhenne et al. 2015).

Inhibition of the GPVI–Collagen Interaction

Glycoprotein VI is a receptor only present on platelets and megakaryocytes, hence making it a very specific target for antithrombotic therapy. The transmembrane region of GPVI is constitutively associated with the Fc receptor γ (FcRγ) chain. This FcRγ chain is essential for the function of GPVI as it is required for GPVI expression, as well as for signal transduction via the immunoreceptor tyrosine-based activation motif (ITAM) and subsequent activation of tyrosine kinase Syk which initiates the downstream signaling cascade (Berlanga et al. 2002). GPVI is present on the platelet in both a monomeric as well as a dimeric form (Dütting et al. 2012). The ligand for GPVI is collagen, with only the dimeric form of GPVI having a high binding affinity. Recent literature however demonstrated that GPVI is also a receptor for polymerized fibrin and that it plays an important role in the thrombus growth and stability by enhancing thrombin generation and the recruitment of new platelets (Mammadova-Bach et al. 2015; Alshehri et al. 2015). Downregulation of platelet reactivity is mediated by GPVI shedding, thus limiting platelet activation during prothrombotic conditions. Shedding of the GPVI ectodomain is mediated by proteolytic activity of ADAM10, a.o. following GPVI cross-linking with and activation of FcRIIa by (auto)antibodies and elevated shear stress (Gardiner and Andrews 2014).

A deficiency in GPVI does not induce a high bleeding risk, again emphasizing its potential as an efficient antithrombotic target. Patients with GPVI deficiency due to genetic mutations or receptor loss via autoantibodies (Moroi et al. 1989; Boylan et al. 2004) only have a mild bleeding disorder; however, platelet aggregation using collagen as an agonist is impaired. Accordingly, mice, in which GPVI function was blocked, present with only a moderately increased tail bleeding time but do have a diminished arterial thrombus formation (Nieswandt et al. 2001; Kato et al. 2003; Bender et al. 2011). These results demonstrate an essential role for GPVI in arterial thrombus formation, however, less so in hemostasis. Additionally, GPVI depletion in mice resulted in reduced brain infarct volume in a transient middle cerebral artery occlusion model for transient ischemic stroke (Kleinschnitz et al. 2007; Kraft et al. 2015). Based on these results, it is expected that targeting GPVI in patients would give a minor bleeding risk, but a strong inhibition of thrombotic processes.

Blockade of GPVI

Targeting of GPVI was done by monoclonal antibodies, Fab fragments, and fusion proteins. Some antibodies can shed

GPVI from the surface of platelets, whereas others only block the interaction with collagen. Monoclonal antibodies from the JAQ family are capable of removing GPVI from the surface of murine platelets, resulting in a GPVI knockout phenotype, associated with protection against lethal thromboembolism with only a mild increased bleeding time (Nieswandt et al. 2001). The antibodies induce a temporary decrease in platelet count at day 1 postinjection, which is completely recovered at day 3 (Nieswandt et al. 2001). The monoclonal antibody F1232 also depletes GPVI from monkey platelets, however, without causing thrombocytopenia (Takayama et al. 2008). This is promising as GPVI apparently can be depleted without the unwanted decrease in platelet count, which is important indeed, as depletion of GPVI by monoclonal antibodies in combination with the absence of integrin $\alpha_2\beta_1$ or with aspirin treatment severely prolonged the bleeding time in mice (Grüner et al. 2004). It might therefore be indicated to monitor the functioning of other receptors before depletion of GPVI as a therapeutic strategy.

Next, Fab fragments were generated from anti-GPVI antibodies. Fab fragments cannot cross-link GPVI receptors or GPVI with the platelet Fc receptor FcRIIa, and hence no longer induce GPVI shedding, but only block its binding site. Administration of Fab fragments OM2 and OM4 inhibited ex vivo aggregation in cynomolgus monkeys and decreased the number of occlusions in a model of cyclic reduction of carotid flow in rats, whereas no increase in bleeding time was observed (Matsumoto et al. 2006; Li et al. 2007). The Fab fragment of mAb 9O12.2 equally inhibited platelet responses toward collagen ex vivo after administration to cynomolgus monkeys (Ohlmann et al. 2008). In a humanized GPVI mouse model, this Fab fragment furthermore prevented lethal thromboembolism and arterial thrombosis (Mangin et al. 2012).

The most advanced GPVI blocking strategy at present is based on the use of a soluble dimeric GPVI-Fc fusion protein. These fusion proteins mimic the dimeric structure of GPVI and therefore compete with platelet GPVI for collagen binding. In vivo, dimeric GPVI-Fc decreased thrombus formation in injured mouse carotid arteries (Massberg et al. 2004; Bültmann et al. 2006) and prevented occlusive thrombus formation and reduced infarct size after ischemic stroke in mice (Goebel et al. 2013), without influencing the bleeding time (Ungerer et al. 2013). Furthermore, the fusion protein reduced myocardial infarct size and resulted in an increased cardiac function after myocardial infarction in mice (Schönberger et al. 2012). The fusion protein was taken to clinical trial as revacept and demonstrated safety and tolerability in a Phase I clinical trial (Ungerer et al. 2011). Currently, a Phase II clinical trial is ongoing where patients with carotid artery stenosis, transient ischemic attacks, amaurosis fugax, and stroke are treated with either revacept plus antiplatelet monotherapy or monotherapy alone (ClinicalTrials.gov identifier: NCT01645306). Readouts include the incidence of microembolic signals, cerebral lesion analysis, clinical end point, and cardiovascular outcomes, and the study is expected to be finalized at the end of 2017. One aspect that might influence the study outcome might be the dosing, as the amount of exposed collagen is not known and not equal in each patient. Even though revacept seems very promising as a future antithrombotic drug, conflicting results were obtained with another dimeric GPVI-Fc fusion protein, where no antithrombotic effects were observed following vigorous ligation injury of the right common carotid artery in the mice (Grüner et al. 2005).

GPVI was also targeted by using EXP3179, an active metabolite of the angiotensin II type 1 receptor antagonist losartan. This drug inhibited GPVI-dependent platelet aggregation and reduced platelet adhesion after acute vessel injury in mice (Grothusen et al. 2007). This effect was achieved by preventing GPVI clustering (Jiang et al. 2015). However, when the drug was administered to patients, no effect on GPVI-dependent platelet aggregation was observed between treated and controls (Jiang et al. 2015). Further targeting the downstream signaling pathway of GPVI is also a possible way of inhibiting GPVI. Syk inhibitor PRT318 targets the GPVI/FcRγ-chain signaling that activates the tyrosine kinase Syk and by this prevents thrombosis in mice (Reilly et al. 2011). However, this inhibitor is not platelet specific as Syk and other components of the downstream signaling are clearly also important in other cells and cellular processes.

Inhibition of Integrin α2β1

Next to GPVI, the other major platelet collagen receptor integrin $\alpha_2\beta_1$ has been investigated as a target for antiplatelet treatment. Integrin $\alpha_2\beta_1$, also known as GPIa/IIa, very late (activation) antigen (VLA)-2/beta, or CD49b/CD29, is a receptor for a number of matrix and non-matrix ligands, including collagens, laminins, tenascins, decorin, E-cadherin, matrix metalloproteinase-1, endorepellin, and several viruses. The $\alpha_2\beta_1$ integrin is expressed on a whole series of cell types, including epithelial and endothelial cells, fibroblasts, specific subsets of leukocytes, and indeed platelets (Madamanchi et al. 2014). It is clear from this list that inhibition of integrin $\alpha_2\beta_1$ might have a considerable number of effects other than merely inhibition of platelet interaction with collagen.

A series of direct and allosteric inhibitors of integrin $\alpha_2\beta_1$ were isolated from snake venoms, hookworms, or herbs (Deckmyn et al. 2012). Also monoclonal antibodies were raised, and peptidomimetics and small molecules were developed (Käpylä et al. 2007; Choi et al. 2007; Nissinen

et al. 2010; Momic et al. 2015). And indeed, in animal models where integrin $\alpha_2\beta_1$ has been inhibited using specific inhibitory monoclonal antibodies or small molecules, a variety of effects was observed including dampening of inflammation responses in wound healing and fibrosis, acceleration of (cancer-related) angiogenesis, or defects in innate immunity (Madamanchi et al. 2014). Vatelizumab, a monoclonal antibody against integrin $\alpha_2\beta_1$, finally is in Phase II clinical trials for acute relapse in multiple sclerosis (ClinicalTrials.gov identifier: NCT02222948).

Nevertheless, targeting $\alpha_2\beta_1$ as an antithrombotic strategy remains attractive, as on the one hand overexpression of $\alpha_2\beta_1$ on human platelets is associated with enhanced thrombotic risk (Moshfegh et al. 1999; Santoso et al. 1999; Carlsson et al. 1999) and, on the other hand, as $\alpha 2\beta 1^{-/-}$ mice have reduced thrombus size in experimental models (He et al. 2003; Kuijpers et al. 2007) with only a mild bleeding tendency (Holtkötter et al. 2002), which was also observed in a patient with an inhibitory autoantibody (Deckmyn et al. 1990). In vivo, evidence for an antithrombotic potential of inhibitors of $\alpha_2\beta_1$ was demonstrated by using small-molecule "compound 15," consisting of a structurally optimized 2,3-diaminoproprionic acid (DAP) moiety combined with benzenesulfonyl-propyl-phenylalanine that significantly reduced thrombus formation in the $FeCl_3$-damaged right carotid artery in mice (Miller et al. 2009). Similarly, BTT-3016, a sulfonamide derivative, was antithrombotic in a mouse model of laser-induced injury to the common carotid artery, with the tail bleeding time prolonged comparable to the effect seen with aspirin (Nissinen et al. 2010).

Discussion and Conclusion

An ideal antiplatelet drug should be able to inhibit platelet adhesion and/or subsequent activation, without inducing major bleeding complications. Blockade of the VWF and/or collagen axis is therefore an attractive therapy, as this would prevent the initial steps of platelet tethering, rolling, and adhesion on the injured vessel wall. The search for clinically applicable compounds has led to the identification of several interesting and promising therapeutic opportunities, from inhibitory antibodies, soluble receptor fragments, and proteins from snake venoms or hematophagous animals to small molecules (summarized in Fig. 2).

The first step of the primary hemostatic response includes the adhesion of plasma VWF to subendothelial collagen, and all available evidence indicates that inhibiting the VWF–collagen interaction represents an effective target to prevent thrombosis without inducing bleeding. However, the inhibitory antibodies did not enter clinical trials, and inhibitory collagen binders isolated from hematophagous animals are not always specific enough for targeted inhibition. These compounds might therefore be less prone for direct clinical application; however, their encouraging effect on preventing experimental thrombosis supports further study.

Next, circulating platelets are captured by immobilized VWF via their GPIbα receptor resulting in tethering and rolling of the platelets. Several interesting compounds have been tested and again showed promising results. With ALX-0081 (caplacizumab) in a clinical trial for thrombotic thrombocytopenic purpura, it will soon become clear whether inhibition of the GPIb–VWF interaction can live

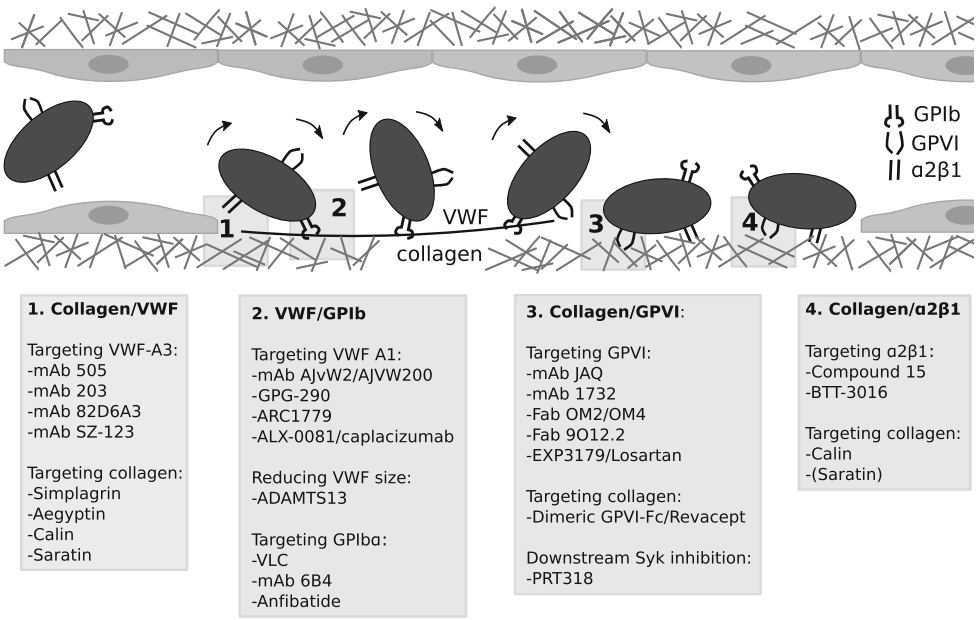

Fig. 2 Overview of inhibitors of platelet adhesion to VWF and collagen for which evidence for in vivo antithrombotic effect is available

up to the expectations with hopeful opportunities for future antithrombotic therapy.

Subsequently, tethering and rolling platelets firmly adhere to collagen via mainly GPVI and integrin $\alpha_2\beta_1$. Based on the results of (pre)clinical studies, GPVI is a promising target for patients with thrombotic complications. The specificity of the GPVI receptor toward the megakaryocyte lineage, the positive results obtained in different studies blocking the GPVI–collagen interaction, and the minimal impact on hemostasis make this an excellent antiplatelet target. With the soluble dimeric GPVI-Fc fusion protein revacept currently in Phase II clinical trial, a future new treatment strategy based on inhibition GPVI–collagen binding might be clinically available soon. As far as integrin $\alpha_2\beta_1$ is concerned, however, relatively limited research has been performed on the potential of targeting this integrin in vivo as an antithrombotic therapy. Nevertheless, even though this receptor is not specific for platelets, it remains an attractive strategy for future research as also here antagonists were able to inhibit thrombus formation without inducing major bleeding complications.

In conclusion, the interactions of VWF and/or collagen with platelets are promising targets for antithrombotic therapy. From preclinical animal studies and clinical patient studies, the accumulated evidence clearly indicates that inhibiting these interactions results in a potent antithrombotic effect without major bleeding episodes. Many of the discussed strategies therefore have the potential to improve the treatment of vascular disease and thrombotic complications in the future.

Take-Home Messages

1. *Inhibition of the VWF–collagen interaction*:
   - The use of monoclonal antibodies inhibiting the VWF–collagen interaction, decreased experimental thrombus formation in vivo; however, they did not enter clinical trials yet.
   - Inhibitors derived from hematophagous animals have potential as antithrombotic medication but sometimes lack specificity.
2. *Inhibition of the VWF–GPIb binding*:
   - Blocking the VWF–GPIb binding using the aptamer ARC1779 or the nanobody ALX-0081 (caplacizumab) shows promising results in the treatment of thrombotic thrombocytopenic purpura.
   - Recombinant ADAMTS13 exerts a protective role in in vivo models of ischemic stroke, myocardial infarction, and thrombotic thrombocytopenic purpura.
3. *Inhibition of the GPVI–collagen interaction*:
   - The soluble GPVI-Fc fusion protein revacept prevents occlusive thrombus formation and currently is being tested in clinical trials.
4. *Inhibition of integrin $\alpha_2\beta_1$ binding to collagen*:
   - Limited research has been performed on targeting this receptor as an antithrombotic therapy.

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Pharmacogenomics of Antiplatelet Drugs

Chiara Cerletti, Benedetta Izzi, Licia Iacoviello, and Giovanni de Gaetano

Abstract

Pharmacogenomics might contribute to the individual response variability to antiplatelet drugs (particularly aspirin and clopidogrel) in patients with ischemic coronary or cerebral vascular disease in terms of both therapeutic and adverse effects. Over the last two decades, a large number of studies and several meta-analyses on the possible genomic regulation of common "polygenic" ischemic cardio- and cerebrovascular diseases have been published. These studies have identified numerous DNA variants as disease or antiplatelet drug susceptibility markers. Some of these DNA variants confer a variable increase in risk for disease, while loss-of-function variants in the hepatic *CYP2C19* system have been reported to be the predominant genetic mediators of clopidogrel and other thienopyridine drug antiplatelet response. However, the overall available data are still somewhat contradictory and do not yet allow to define a clear role for pharmacogenomics in providing effective, reliable, personalized antiplatelet therapy: neither clear conclusions nor definite guidelines are available on the clinical advantages of pharmacogenetic testing before prescribing antiplatelet drugs. In the near future, the relative role of additional rare polymorphisms, structural variants and tissue-specific epigenetic features of the human genome will hopefully be defined as significant contributors to the pathogenesis of cardio- and cerebrovascular disease and of individual patient's response to antiplatelet drugs.

Introduction

Pharmacogenomics is the study of how variations in the human genome affect the response to medications. This term substitutes the older "pharmacogenetics", created from the words "pharmacology" and "genetics", to indicate the intersection of pharmaceuticals and genetics. It describes the possibility to analyze multiple genes simultaneously, rather than one at a time, on a large scale, often by a genome-wide approach, thanks to the availability of new genomic technologies.

Pharmacogenomics has been implicated in the individual response variability to antiplatelet drugs (particularly aspirin and clopidogrel) in patients with ischemic vascular disease, mainly coronary and cerebrovascular disease, such as myocardial infarction and stroke, in terms of both therapeutic and adverse effects. A large number of studies and several meta-analyses have been published on this topic, but, until recently, neither clear conclusions nor definite guidelines are available on the clinical advantages of pharmacogenetic testing before prescribing antiplatelet drugs. In this review, we shall mainly refer to meta-analyses of randomized clinical studies. Some relevant open questions will also be summarized and discussed.

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Variability of Functional Platelet Response to Antiplatelet Drugs: Early Observations

Despite the efficacy of antiplatelet therapy in preventing ischemic cardio- and cerebrovascular events, shown by hundreds of randomized controlled clinical trials and confirmed by several large meta-analyses (Antithrombotic Trialists' Collaboration 2002; Antithrombotic Trialists' ATT Collaboration et al. 2009), many patients still experience ischemic events while on standardized antiplatelet treatments. This has spurred a large debate on the variability of platelet response, the optimal monitoring of antiplatelet therapy and the relationship between ex vivo measurements of platelet function and clinical outcomes (de Gaetano and Cerletti 2003, 2007; Michelson et al. 2005; de Gaetano et al. 2008).

The question was addressed whether measuring individual platelet response to antiplatelet therapy could be useful to predict clinical outcomes and to modulate antiplatelet drug treatment for better achieving prevention or treatment of thrombosis in otherwise "nonresponder" subjects (de Gaetano and Cerletti 2003). A widespread effort was thus made to try to predict vascular events, therapeutic response and clinical outcomes by platelet function tests.

During the past decades, indeed, clinical trials on antiplatelet drugs had been (and still are) planned and performed on patients enrolled without any previous measurement of their basal platelet function, or of their platelet response to the drug under trial, as measured by laboratory tests. Thus clinical end points could not be correlated to any laboratory parameter of platelet function.

To contribute to thrombosis—it was argued—platelets do not need to hyper-aggregate; it is sufficient that they simply aggregate. This view was shared by many investigators, as confirmed by the fact that no platelet marker was utilized to monitor or predict the risk of vascular events in different clinical conditions (Patrono et al. 1984; Van de Loo 1989; Cortellaro et al. 1992).

However, a variable platelet response to aspirin had already been reported together with the first observation that this drug could interfere with the haemostatic system (de Gaetano 2001). Indeed, more than half a century ago, Quick (1966) proposed an "aspirin tolerance test" based on his observation that not all patients with von Willebrand disease showed a similar prolongation of the bleeding time after aspirin. When, a few years later, the inhibitory effect of aspirin on platelet aggregation was described, it appeared that only some parameters of platelet function such as the second irreversible wave of aggregation induced by ADP or adrenaline, but not others, such as the aggregating response to thrombin, could be prevented by aspirin (Weiss et al. 1968; O'Brien 1968). Shortly thereafter, Smith and Willis (1971) reported that aspirin suppresses platelet prostaglandin production but not platelet aggregation induced by thrombin. However, the observation that TxA2 generation in platelets could be substantially inhibited by any dose of aspirin and the availability of an easy and reliable method for measuring TxB2 in serum (Patrignani et al. 1982) focused the attention on a biochemical parameter endowed with relatively low variability rather than on the evaluation of the multifaceted and variable platelet function.

Moreover, it was observed already in 1968 that in some subjects single doses of aspirin as low as 150 mg, much lower than the anti-inflammatory doses used at that time, were able to variably suppress platelet aggregation and serotonin release for several days; concomitantly, aspirin, but not salicylate, was found to be active, suggesting a role for the irreversible acetylation of some intra-platelet proteins (later on identified as the enzyme cyclo-oxygenase-1, COX-1) in the mechanism of the long-lasting action of aspirin (de Gaetano et al. 1985).

The observation that other non-steroidal anti-inflammatory drugs, such as indomethacin, were platelet inhibitors comparable to aspirin, though short lasting, suggested the existence of a putative platelet common binding site, different from the acetylation site, accessible on COX-1 to all these anti-inflammatory compounds (Cerletti et al. 1982a, b; Livio et al. 1982). The interaction at the level of the binding site of COX-1 between non-steroidal anti-inflammatory drugs, such as indomethacin or ibuprofen, and aspirin was also documented and confirmed (de Gaetano et al. 1985; Catella-Lawson et al. 2001). The possibility that salicylate, rapidly accumulating in blood during chronic administration of aspirin, could interfere with the antiplatelet effect of the parent compound and induce a variable response to the latter was also proposed and demonstrated by different techniques (Dejana et al. 1981, 1985; Cerletti et al. 1982a, b). Functional and modelling studies recently extended to different plant-derived polyphenols (such as gallic acid, resveratrol, quercetin) that have the capacity to bind to platelet COX-1 and to influence the effect of aspirin on platelet function (Crescente et al. 2009). Nutritional compounds of different chemical nature may thus interfere with antiplatelet drugs and partially explain interindividual variability of inhibition of platelet response; but this interesting approach is at the moment of theoretical interest only.

The existence of "responders" and "nonresponders" to antiplatelet drugs other than aspirin is not a recent finding either. In 1974, indomethacin, a non-steroidal anti-inflammatory drug given at that time to patients with chronic glomerulonephritis, reportedly induced a variable inhibition of platelet aggregation tests: stronger inhibition of platelet function was significantly associated with higher reduction of urinary excretion of protein and fibrinogen-/fibrin-related antigen (de Gaetano et al. 1974). A few years later, variability of functional platelet response to TxA2-synthase

inhibitors was also reported by several groups (Heptinstall and Fox 1983; Bertelé et al. 1984; Gresele et al. 1988).

The apparent discrepancy between studies based on platelet function test measurements reporting "aspirin resistance" and the positive results of clinical trials of aspirin prophylaxis in high-risk patients initially pointed to the limitations and unreliability of platelet function studies (Patrono 2003; Santilli et al. 2009). Such an apparent discrepancy was subsequently reconciled by acknowledging the biological and clinical relevance of platelet function variability and the potential limitations of randomized clinical trials performed on large selected populations when extrapolated to individuals. Clinical trials were compared to a kind of "epidemiological night" where all patients look black, as it was not possible to identify in advance the small number of "responders" in terms of reduced clinical outcomes (de Gaetano et al. 2003).

However, if someone would ask: "Should we treat patients with aspirin or other antiplatelet drugs to prevent coronary heart disease or stroke?" the right answer should still be: "Rely upon the results of randomized controlled clinical trials and meta-analyses on the antithrombotic clinical efficacy of antiplatelet drugs" (de Gaetano and Cerletti 2007).

Residual On-Treatment Platelet Response to Aspirin

In the presence of sufficient aspirin dosing and compliance, failure to inhibit TxA2-dependent platelet activation tests appears to be rather uncommon. During the last years, however, "aspirin resistance", better defined as residual on-treatment platelet response measured by platelet function tests other than TxB2 measurement, was reported to be significantly correlated with the lack of clinical response in patients with cardiovascular disease (Freedman 2006; Crescente et al. 2008; Sofi et al. 2008). Advantage was mainly taken from some newly developed point-of-care tests of platelet function. Different meta-analyses reported high prevalence of residual on-treatment platelet response to aspirin at a population level. Among aspirin-treated subjects, a much lower prevalence of hypo-responsiveness for TxA2-dependent assays was found (approximately 6 % for arachidonic acid stimulation), while a higher number of subjects appeared to be lower responders to stimulation of TxA2-independent pathways (PFA-100, 27–33 %) (Crescente et al. 2008, 2011; Krasopoulos et al. 2008). Data from a meta-analysis of 20 studies, totalling 2930 patients, indicate that in patients with cardiovascular disease taking aspirin at doses 75–325 mg daily, nonfatal or fatal cardiovascular events occurred in 39 % of patients with residual on-treatment platelet response to aspirin (assessed by various assays) compared to 16 % events observed among aspirin-responder patients (OR = 3.85, 95 % CI 3.08–4.80) (Krasopoulos et al. 2008). Residual platelet activation in aspirin-treated patients was associated in other studies with an eightfold excess in the occurrence of future cardiovascular events (Frelinger et al. 2006). In a systematic review of eight studies comprising 847 subjects, aspirin nonresponders, as identified by PFA-100, were more likely to have vascular events than aspirin responders (relative risk (RR) 1.63; 95 % CI 1.16–2.28) (Crescente et al. 2008), a finding confirmed in a large meta-analysis of 19 studies comprising 3003 patients (Crescente et al. 2009).

Genetic Variants and Variability of Functional Platelet Response to Aspirin

The functional response of platelets to aspirin appears to be heritable. Studies on aspirin pharmacogenetics tried to identify individuals with different sensitivity to the drug, allowing the recognition of possible genetic regulation of platelet response variability to aspirin and of the susceptibility to atherothrombotic events during aspirin treatment (Freedman 2007; Fitzgerald and Pirmohamed 2011).

Genetic variation in the molecules responsible for platelet activation might be an important potential contributor to the adequacy of the antiplatelet action of aspirin. Table 1-(A) reports the genes and their polymorphic variants shown to be involved in the response to aspirin. Thus far, however, candidate gene studies have not provided consistent evidence for a critical gene-aspirin response relationship. The *COX-1* gene region appears to be highly polymorphic: in particular, studies on C50T, the most extensively investigated candidate gene polymorphism, gave conflicting results; indeed, some studies showed no association with aspirin response, while studies which have shown an association with lower response to aspirin were not correlated with clinical outcomes (Lepäntalo et al. 2006; Clappers et al. 2008; Voora et al. 2011).

Previous studies had focused on polymorphisms of genes encoding for different platelet membrane glycoproteins in small groups of aspirin "nonresponder" patients in terms of platelet aggregation inhibition: platelets from individuals homozygous for the PlA1 allele, encoding for the glycoprotein (Gp)IIIa, appeared indeed to be less sensitive to the inhibitory action of low-dose (160 mg) aspirin. The relationship between GpIIIa and the antiplatelet mechanism of aspirin is however obscure. Goodman et al. (2008) summarized 31 small observational studies, assessing the association between several genetic polymorphisms of candidate genes and clinical response to aspirin. In that review, 50 polymorphisms in 11 genes were investigated in 2834 subjects, either healthy or with cardiovascular disease: the

Table 1 Gene polymorphisms and functional or clinical responses to antiplatelet drugs from main studies

Gene	Variant allele	Outcome	Conclusion
(A) Aspirin			
GPIIIa	PlA1A2	Clinical end points (MACE, ST); surrogate end points	Uncertain association
GPIa	C807T	Clinical end points (MACE); surrogate end points	No association
GPIb	rs2243093	Clinical end points (MACE)	
GPVI	rs1613662	Clinical end points (MACE)	
$P2Y_{12}$	Haplotype H1/H2	Surrogate end points	
$P2Y_1$	A1622G	Surrogate end points	
COX-1	rs3842787; A842G;C50T	Clinical end points (MACE); surrogate end points	
COX-2	G-765C	Clinical end points (MACE)	Probable
PEAR1	rs2768759; rs12041331	Clinical end points (MACE); surrogate end points	Uncertain association
Apo(a)	Rs3798220	Clinical end points (MACE)	Probable
(B) Clopidogrel			
CYP2C19	Loss-of-function allele (*2, *3,*4, *5, *6) Gain-of-function allele (*17)	Clinical end points (MACE, ST); surrogate end points	Uncertain association
CYP2C9	Loss-of-function allele (*2, *3)	Clinical end points (MACE, ST)	No association
CYP3A4	*1B IVS10 + 12A	Clinical end points (ST); surrogate end points	No association
CYP3A5	*3, *2	Clinical end points (MACE, ST); surrogate end points	Uncertain association
CYP2B6	Reduced function alleles	Clinical end points (MACE); surrogate end points	No association
ABCB1	C3435T; C1236T, G2677T/A	Clinical end points (MACE, ST); surrogate end points	No association
PON1	Q192R	Clinical end points (MACE); surrogate end points	No association
GPIIIa	PlA1/A2	Clinical end points (MACE); surrogate end points	No association
$P2Y_{12}$	G52T; C34T; T744C; rs16846673; rs6798347; rs9859552; rs6801273; rs9848789; rs6787801; rs6785930; rs2046934; Haplotype H1/H2; Haplotype F vs. A	Clinical end points (MACE, ST); surrogate end points	Probable for the haplotypes; no association for other polymorphisms
CYP1A2	Reduced function allele	Clinical end points (MACE); surrogate end points	No association
$P2Y_1$	A1622G	Clinical end points (ST)	No association
GP1a	C807T	Clinical end points (MACE); surrogate end points	No association
PAR1	rs618753; rs6450105	surrogate end points	No association
PEAR1	rs12041331; rs11264579; rs41299597; rs822442; rs41273215; rs3737224	surrogate end points	No association
COX-1	A-842G	Clinical end points (MACE, ST)	No association
(C) Prasugrel			
CYP2C19	Loss-of-function alleles	Clinical end points (MACE, ST); surrogate end points	No association
CYP2C9	Loss-of-function alleles	Clinical end points (MACE)	No association
CYP2B6	Loss-of-function alleles	Clinical end points (MACE)	No association
CYP3A5	Loss-of-function alleles	Clinical end points (MACE)	No association
CYP1A2		Clinical end points (MACE)	No association
ABCB1	C3435T; G2677T/A; C1236T	Clinical end points (MACE); surrogate end points	No association
PON1	Q192R	Clinical end points (MACE, ST) surrogate end points	No association
(D) Ticagrelor			
CYP2C19	Loss-of-function alleles	Clinical end points (MACE, ST)	No association
ABCB1	C3435T	Clinical end points (MACE, ST)	No association

MACE major adverse cardiovascular events, *ST* stent thrombosis

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PlA1/A2 SNP present in the GpIIIa receptor expressed on the platelet surface was the most investigated, followed by the C807T polymorphism present on the GpIa receptor gene, the A842G and C50T SNPs in the *COX-1* gene, the H1/H2 SNP in the $P2Y_{12}$ receptor gene and the A1622G SNP in the $P2Y_1$ gene. However, the pharmacogenetic studies reviewed in that meta-analysis only considered aspirin resistance as defined by laboratory tests of platelet function, with large method variations from one study to another. There was a significant association between the PlA1/A2 molecular variant and aspirin resistance in healthy subjects (OR 2.36; 95 % CI 1.24–4.48). Such an association was no more apparent in patients with cardiovascular disease (OR 0.92; 95 % CI 0.65–1.30) and when combining the two subgroups (OR 1.14; 95 % CI 0.84–1.54). In addition, the observed association between platelet response to aspirin and the PlA1/A2 polymorphism was only seen when measuring platelet function by aggregometry but not by PFA-100 (Goodman et al. 2008). In a study not included in the review by Goodman et al. (2008), in patients receiving low-dose aspirin and ticlopidine with successful percutaneous coronary intervention, a significant association with the increased risk of the composite outcome of death or myocardial infarction was shown in carriers of the PlA2/A2 genotype (Kastrati et al. 2000).

Failure to show any consistent association between polymorphisms in platelet protein genes and variability of platelet response to aspirin inhibition is possibly due to inadequate assessment of phenotype or to the fact that the contribution of each single gene variant to the overall phenotypic variance was too low and/or the population size too small.

More recent studies, reviewed by Yasmina et al. (2014), also showed conflicting results: indeed, while the PlA1/A1 genotype was associated with a twofold increased risk of high platelet reactivity as assessed by PFA-100 (Abderrazek et al. 2010), patients with the PlA2/A2 genotype had higher ADP-induced platelet aggregation than patients with PLA1/A1 or PLA1/A2 genotypes (Lordkipanidzé et al. 2011); in the latter study no association with arachidonic acid-induced platelet aggregation or urinary concentration of 11-dehydro TxB2 was observed. Lately, PlA polymorphism associated with in vitro aspirin resistance was unrelated to clinical outcomes, death or myocardial infarction, in patients with coronary artery disease, who reported regular aspirin use (Voora et al. 2011).

Studies of other polymorphisms related to either platelet function (GPIIIa, GPIa, GPIb, GPVI, $P2Y_1$, $P2Y_{12}$, CYP2C19, TBXA2R) or inhibition of the pharmacological targets for aspirin (COX-1, COX-2) failed to show any consistent association with the effect of aspirin or clinical outcomes, with the possible exception of *COX-2* genetic polymorphisms, as the *PTGS2* G765C mutation (rs20417). The latter was more frequent in non-aspirin- compared to aspirin-sensitive patients in a cohort of 674 Chinese hypertensive patients (Xu et al. 2012) and was associated with a reduced risk of myocardial infarction and interaction with aspirin use (P for interaction = 0.03) (Lemaitre et al. 2009). In the genetic sub-study of the International Verapamil SR/Trandolapril Study (INVEST), a genome-wide analysis showed a strong association between SNPs on chromosome 1q23 and post-dual antiplatelet therapy. Moreover, in the presence of clopidogrel, genotyping revealed the rs12041331 A-allele in the platelet endothelial aggregation receptor-1 (*PEAR1*) gene to be a strong determinant of collagen-induced platelet aggregation in response to aspirin. Surprisingly, the same rs12041331 A-allele, which was associated with lower post-aspirin collagen-stimulated platelets, led to increased cardiovascular events in percutaneous coronary intervention patients on dual antiplatelet therapy and in an independent population with stable coronary artery disease treated with aspirin alone (Lewis et al. 2013).

Ten-year follow-up data from the Women's Genome Health Study (WGHS), a large population-based cohort of women randomly allocated to aspirin or vitamin E, compared with placebo, indicated that the common polymorphism rs4680 of catechol-O-methyltransferase (*COMT*), a key enzyme in catecholamine metabolism, is associated with incident cardiovascular disease in placebo-treated women and that this association is abolished by treatment with aspirin (Hall et al. 2014). How aspirin could modify cardiovascular disease protection associated with COMT is not known, but candidate mechanisms include effects on platelet function or homocysteine levels and are in agreement with the observation that the differential response to aspirin therapy in a variety of settings is a heritable trait.

Faraday et al. (2007) performed genome-wide linkage (GWL) and association analyses on the same samples through analysis of 37 agonist-induced platelet phenotypes, collected from 1880 asymptomatic subjects before and 14 days after treatment with 81 mg/day aspirin. Eleven patterns of similar platelet function phenotypes, pooled with a principal component analysis, showed suggestive evidence for linkage across 14 regions in the genome, with post-aspirin treatment phenotypes showing higher linkage than in pre-aspirin samples.

In conclusion, aspirin-induced platelet function inhibition may vary at an individual level due to still undefined genetic control. However, the relevance of such a genetic control still remains to be established and seems in any case to be relatively poor.

Variability of Functional Platelet Response to Clopidogrel

For several years it was believed that the variable individual response to antiplatelet therapy was a prerogative of aspirin and a great boost was given to the use of other molecules with other platelet targets, such as the blockade of the ADP receptor $P2Y_{12}$, by clopidogrel and other thienopyridines.

Clopidogrel, a second-generation thienopyridine, is absorbed as a prodrug and converted to its active thiol metabolite by hepatic cytochrome (CYP) P450 enzymes, encoded by a highly polymorphic gene. The prodrug clopidogrel is absorbed via ATP-binding cassette efflux transporters located in the apical membrane of the intestinal mucosa encoded by the multidrug resistance gene *MDR* (*ABCB1*). The majority of the absorbed prodrug is inactivated by hepatic esterases and lacks any antiplatelet activity. Less than 15 % of the absorbed prodrug is further metabolized by two sequential oxidative steps through the hepatic CYP450 system, to generate the active thiol metabolite, which targets and irreversibly inhibits the ADP $P2Y_{12}$ receptor. Figure 1 summarizes the metabolic pathways of

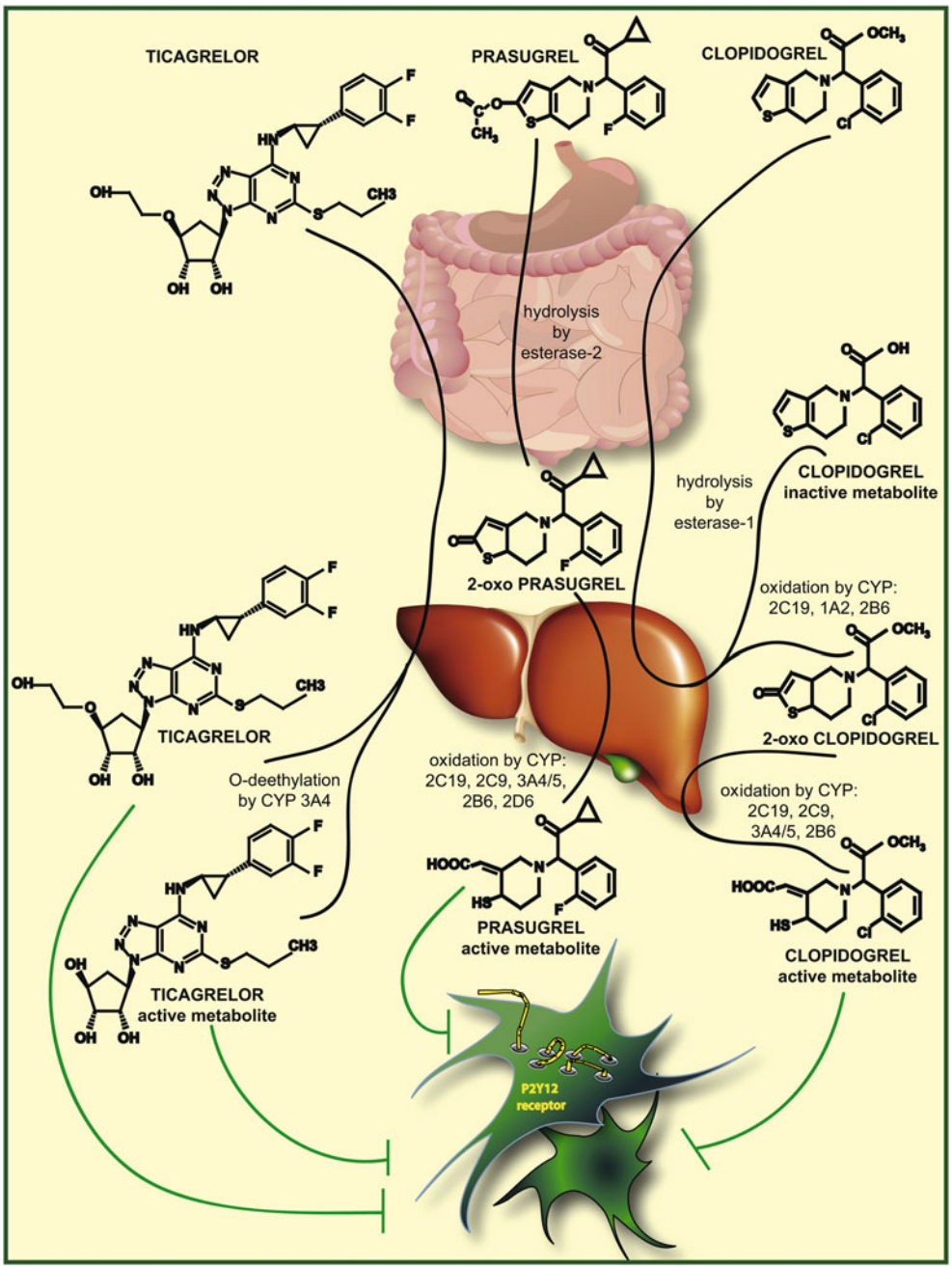

Fig. 1 Chemical structures and schematic metabolic pathways of $P2Y_{12}$ receptor inhibitors. *ADP* adenosine diphosphate, *CYP* cytochrome P450. Reproduced with permission from Siller-Matula et al. (2013)

$P2Y_{12}$ receptor inhibitors (Siller-Matula et al. 2013). Among the CYP isoenzymes, so far identified as having a relevant metabolic role, the polymorphically expressed *CYP2C19* affects both metabolic steps of clopidogrel active metabolite generation and therefore plays a dominant role in this process (Kazui et al. 2010).

The clopidogrel response variability, hypothesized on theoretical grounds (de Gaetano et al. 2002), was first described by Gurbel et al. (2003) followed by several other confirmatory studies, recently reviewed by Cattaneo (2011) and by Bernlochner and Sibbing (2012).

A regimen including both aspirin and clopidogrel has been introduced and extensively used in the secondary prevention of cardio- and cerebrovascular events in patients with previous stroke, myocardial infarction or peripheral arterial disease. The efficacy of clopidogrel in addition to aspirin was shown in the CURE trial, conducted in 12,562 patients with acute coronary syndrome without ST-segment elevation, randomized to aspirin or aspirin plus clopidogrel. The dual therapy reduced the composite end point of cardiovascular death, myocardial infarction or stroke (Yusuf et al. 2001) and is recommended by recent guidelines (Amsterdam et al. 2014; Authors/Task Force Members et al. 2015).

While aspirin variability remains an uncontrolled variable not or only minimally based on genetic polymorphisms, clinical evidence suggests that reduced responsiveness to clopidogrel is the main cause of the reduced efficacy of dual antiplatelet therapy (Peace et al. 2008).

Data from a meta-analysis of 14 studies recruiting 4564 subjects show that among patients with coronary artery disease, who underwent percutaneous coronary intervention, the risk of death and/or thrombotic recurrences increased by about sixfold in those with a poor response to clopidogrel treatment (Sofi et al. 2010).

Criteria to define clopidogrel non-response are numerous and discrepant, some with obvious limits; it is based on either relative or absolute differences in platelet aggregation before and after clopidogrel, or arbitrary cut off points, or two standard deviations from the mean to define low responders. It is therefore often difficult to establish a consensus from literature data on the incidence of clopidogrel non-response (Bonello et al. 2010). The 2013 update of the consensus on the definition of on-treatment platelet reactivity to ADP questioned whether treatment modifications based on the results of current platelet function test platforms can actually influence clinical outcomes. In fact, prospective clinical trials were unable to show any relevant clinical benefit of platelet function tests. In addition, the observation that low-on-treatment platelet reactivity to ADP is associated with a higher risk of bleeding, suggested that a therapeutic window should be considered for $P2Y_{12}$ inhibitor therapy (Tantry et al. 2013).

Genetic Variants and Variability of Functional Platelet Response to Clopidogrel

The primary cause of variability in response to clopidogrel lies in its pharmacokinetics, being largely dependent on the bioavailability of its active metabolite. Multiple candidate gene variants, involved in clopidogrel absorption and activation and in inhibition of the $P2Y_{12}$ receptor, have been shown to correspond to either high or low platelet reactivity. Table 1-(B) reports the genes and their polymorphic variants involved in clopidogrel response. A loss-of-function polymorphism of the *CYP2C19* polymorphism was first demonstrated in acute coronary syndrome patients undergoing percutaneous coronary intervention on clopidogrel: the *2 allele was associated with high residual platelet function, and the *2*2 homozygous subjects had significantly higher platelet aggregation than the *1*2 heterozygotes (Giusti et al. 2007). This finding has been confirmed in other clinical settings (Giusti et al. 2010; Gurbel et al. 2012; Krishna et al. 2012) and most importantly associated with the occurrence of major adverse clinical events (Mega et al. 2009a; Shuldiner et al. 2009; Simon et al. 2009), in particular stent thrombosis in patients treated with clopidogrel (Gurbel et al. 2011).

The results of studies on the association between *CYP2C19* loss-of-function alleles and clinical efficacy of clopidogrel have been reported in several meta-analyses, with apparently discordant conclusions; these have been carefully analysed and critically discussed in a recent systematic review by Osnabrugge et al. (2015): 11 meta-analyses were considered, published in the frame of 2 years, including 30 primary studies, not all included in all meta-analyses (Table 2). Besides several differences among the studies selected for those meta-analyses, such as inclusion or not of conference abstracts, check of quality of primary studies and methods used, all 30 primary studies separately addressed composite clinical end points and stent thrombosis. The results and conclusions of the meta-analyses on the association between *CYP2C19* loss-of-function alleles and clinical efficacy of clopidogrel were discordant: eight out of 11 reported a statistically significant association, with mean effect size ranging from 1.26 to 1.96. Within these positive meta-analyses, five concluded that there was association between loss-of-function alleles and the clinical end point, two stated that the association was possible, and one concluded that this was not proven because of publication bias. Within the other three meta-analyses out of 11, two found no statistically significant pooled effects, and one did not pool the data because of the heterogeneity of the studies analysed. On the other hand, all 11 meta-analyses reported a statistically significant association between *CYP2C19* loss-of-function alleles and stent thrombosis,

Table 2 Characteristics of meta-analyses on CYP2C19 genotype and risk of adverse clinical outcomes in clopidogrel users

	Definitions		Number of studies	
First author, year of publication	Clinical end point	Stent thrombosis	Clinical end point	Stent thrombosis
Hulot, 2010	Death, MI, stroke, urgent vascularization	Definite or probable according to ARC	10	4
Jin, 2011	Clinical adverse events	Not defined	8	5
Mega, 2010b	CV death, MI, stroke	Definite or probable according to ARC	9	6
Sofi, 2011	Death, MI, stroke, unstable angina, stent thrombosis, recurrent ischemia	Not defined	7	4
Zabalza, 2012	CV death, MI, stroke, unstable angina, recurrent ischemia	Definite or probable according to ARC	11	7
Liu, 2011	Major adverse clinical events (not specified)	Not defined	18	9
Bauer, 2011	Death, MI, stroke	Definite: according to ARC Probable: only if reported as composite outcome Possible: not considered	12	9
Holmes, 2011	Death (any cause), CHD, stroke, stent thrombosis, revascularization, hospitalization for ACS	Not defined	26	14
Jang, 2012	Death, MI, stroke, stent thrombosis	Definite or probable according to ARC	16	10
Singh, 2012	Major adverse clinical events (not specified), CV death, MI, stent thrombosis, stroke, major bleedings	Definite or probable	14	6
Yamaguchi, 2013	Death, MI, stroke, stent thrombosis, revascularization	Not defined	7	5

ACS acute coronary syndrome, *ARC* Academic Research Consortium, *CHD* coronary heart disease, *CV* cardiovascular, *MI* myocardial infarction
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with mean effect size ranging from 1.77 to 3.82. Two meta-analyses downgraded this evidence, one concluding that the association was possible and the other that there was not, due to the presence of heterogeneity and publication bias (Osnabrugge et al. 2015).

In 2010, the US Food and Drug Administration added a boxed warning to the clopidogrel label, with an alert to clinicians on the reduced effectiveness of clopidogrel in patients who are poor metabolizers of the drug, leaving however the decision to use genetic tests to the decision of physicians (FDA Drug Safety Communication 2015).

The authors of the systematic review mentioned above, on the basis of a re-evaluation of all the available meta-analyses, concluded that the FDA recommendation of genotyping for individualized antiplatelet therapy is currently not evidence based.

Similar conclusions were obtained by the results of the GRAVITAS, ARCTIC and TRILOGY-ACS trials, in which bedside-tested platelet reactivity did not result in any clinical benefit (Price et al. 2011; Collet et al. 2012; Gurbel et al. 2012).

The efficacy and safety of intensified antiplatelet therapy (peri-procedural GPIbIIa inhibitor infusions, repeated loading dose or increased maintaining dose of clopidogrel or use of prasugrel) tailored on the basis of individual platelet reactivity tests have been evaluated in prospective studies, reviewed by Aradi et al. (2013a). The conclusions reached by this meta-analysis including ten studies involving 4213 patients undergoing percutaneous coronary intervention are that the intensified protocol was associated with a significant reduction in cardiovascular mortality, stent thrombosis and myocardial infarction, without any difference in major bleeding events. However, the net clinical benefit of the intensified treatment versus the standard one significantly depended on the risk of stent thrombosis with a standard dose of clopidogrel.

A recent prospective proof-of-concept trial randomized 200 patients undergoing percutaneous coronary intervention to either point-of-care genotyping and subsequent personalized treatment or standardized clopidogrel treatment. A significantly lower on-treatment platelet reactivity was observed in the *CYP2C19* loss-of-function allele carriers than in the standard treated patients (Roberts et al. 2012). However, in the subgroup of the ARCTIC study, genotyping the results added little to the pharmacodynamic data to adjust antiplatelet therapy (Collet et al. 2015). These findings support the conclusion that there might still be room for optimization of antiplatelet therapy by genotyping. In fact, negative trials on the use of personalized therapy largely suffer for inappropriate study design, so that the patients who might benefit from personalized antiplatelet therapy on the basis of pharmacogenomics require further classification.

Other genes (*ABCB1, CES1, PON1* and *P2RY12*) encoding for proteins involved in pharmacokinetics and pharmacodynamics of clopidogrel, listed in Table 1-(B),

have been critically discussed elsewhere (Perry and Shuldiner 2013), but understanding their clinical relevance requires further studies.

Variability of Functional Platelet Response to Prasugrel and Non-thienopyridine Derivatives

Prasugrel, a new thienopyridine, is rapidly hydrolysed by carboxylesterases to a thiolactone, further metabolized to an active metabolite via one-step CYP450 metabolism. The active metabolite irreversibly binds to the $P2Y_{12}$ receptor. Prasugrel's active metabolite requires therefore a reduced contribution from CYP2C19 than clopidogrel (see Fig. 1).

The non-thienopyridine derivatives ticagrelor, cangrelor and elinogrel, on the other hand, do not require hepatic conversion and directly and reversibly bind to the $P2Y_{12}$ ADP receptor in a concentration-dependent manner (Cattaneo 2010).

The efficacy of prasugrel was shown to be greater than clopidogrel in the prevention of ischemic events in patients undergoing percutaneous coronary intervention for ST-elevation myocardial infarction (TRITON-TIMI 38) (Montalescot et al. 2009); increased major bleeding compared to clopidogrel was only reported after coronary artery bypass grafting (CABG). The drug use was approved by FDA and EMA in patients with acute coronary syndrome undergoing percutaneous coronary intervention to reduce thrombotic cardiovascular events; it is not generally recommended in elderly patients (>75 years) or in patients with body weight <60 kg or with a history of stroke or transient ischemic attack.

A meta-analysis of randomized, controlled clinical trials comparing the clinical impact of clopidogrel with placebo or prasugrel/ticagrelor versus clopidogrel, comprising a total of 107,473 patients, included cardiovascular death, myocardial infarction, total stroke and intracranial haemorrhage as outcome measures (Aradi et al. 2013b). Clopidogrel reduced the risk of cardiovascular death, myocardial infarction and stroke, without influencing the risk for intracranial haemorrhage. Treatment with prasugrel/ticagrelor provided additional benefit over clopidogrel regarding cardiovascular mortality and myocardial infarction but demonstrated no advantage on stroke and intracranial haemorrhage. Increased potency of $P2Y_{12}$ receptor inhibition is associated with a decreased risk in cardiovascular death and myocardial infarction but not in stroke with respect to clopidogrel.

These results have been confirmed and extended by two recent meta-analyses: Chen et al. (2015) included nine studies involving 25,214 patients and found that the risks of major adverse clinical events outweighed those of major bleeding (OR 7.48, 95 % CI 3.75–14.94, $p < 0.0001$, random effects) and of minor bleeding (OR 3.77, 95 % CI 1.73–8.22, $p = 0.009$, random effects), results corroborated in a standard-dose clopidogrel subgroup analysis (OR 7.46, 95 % CI 3.54–15.68, $p < 0.0001$, and OR 6.44, 95 % CI 2.80–14.80, $p < 0.0001$, random effects, respectively). Despite the increased risk of bleeding associated with prasugrel treatment compared with clopidogrel, the risk of major adverse clinical events far outweighed the risk of bleeding. The meta-analysis by Bavishi et al. (2015) compared the results of randomized clinical trials with newer oral $P2Y_{12}$ inhibitors (prasugrel or ticagrelor) to clopidogrel in 31,470 patients with non-ST-elevation acute coronary syndrome. The primary outcome was a composite of cardiovascular death, myocardial infarction and stroke. Newer oral $P2Y_{12}$ inhibitors significantly decreased major adverse clinical events (RR 0.87, 95 % CI 0.80–0.95) and myocardial infarction (RR 0.85, 95 % CI 0.75–0.96) and showed a trend towards reduction of cardiovascular death (RR 0.89, 95 % CI 0.71–1.01). There was a significant increase in major bleedings (RR 1.27, 95 % CI 1.07–1.50) and major or minor bleedings (RR 1.20, 95 % CI 1.02–1.42). Results were largely similar when stratified by ticagrelor versus prasugrel (p interaction >0.05) except for increased TIMI major/minor bleedings with prasugrel than ticagrelor (p interaction = 0.01).

In conclusion, in patients with non-ST-elevation acute coronary syndrome, newer oral $P2Y_{12}$ inhibitors decrease major adverse clinical events and myocardial infarction at the expense of a significant increase in the risk of bleeding. Treatment of 1000 patients with newer oral $P2Y_{12}$ inhibitors will prevent 16 major adverse clinical events and 13 myocardial infarctions at the expense of increase in six major bleeding events (Aradi et al. 2013b).

In 2014, cangrelor was approved by the Food and Drug Administration for two indications: for the reduction of thrombotic cardiovascular events (including stent thrombosis) in the setting of percutaneous coronary intervention in patients with coronary artery disease and for thrombotic events (such as stent thrombosis) in the setting of bridging therapy in patients with acute coronary syndrome or with stents who are at increased risk when oral $P2Y_{12}$ therapy is interrupted because of surgery (Lhermusier et al. 2015).

Elinogrel is a novel competitive, reversible ADP-receptor antagonist available in oral and intravenous formulation. Additional treatment with elinogrel showed advantages over clopidogrel, including more rapid, less variable and more complete inhibition of platelet function without significantly increased bleeding complications (Müller et al. 2012).

No data are presently available on the pharmacogenetics of these novel inhibitors of $P2Y_{12}$ receptor activation.

Genetic Variants and Prasugrel and Ticagrelor Response

Table 1-(C and D) reports the main genes and their polymorphic variants involved in prasugrel and ticagrelor response, respectively. CYP2C19 is one of the enzymes, which contribute to the metabolism of prasugrel, although to a smaller extent than that seen with clopidogrel. Prasugrel treatment is more effective than clopidogrel in carriers of the loss-of-function allele of *CYP2C19* but has an apparently higher risk of bleeding (Alexopoulos et al. 2011; Grosdidier et al. 2013); on the other hand, carriers of the wild-type genotype of *CYP2C19* show similar efficacy and safety of prasugrel and clopidogrel treatment (Sorich et al. 2010; Cuisset et al. 2012).

No association has been observed between genetic polymorphisms of other metabolizing enzymes (*CYP2C9, CYP2B6, CYP3A5 and CYP1A2*) or efflux transporters (*MDR1*) and pharmacokinetics, pharmacodynamics and clinical outcomes with prasugrel (Mega et al. 2009b, 2010a).

A very recent publication reported the results of *PON1* genotyping of 2922 patients with an acute coronary syndrome undergoing percutaneous coronary intervention, randomized to treatment with clopidogrel or prasugrel in the TRITON-TIMI 38 trial*:* there was no association between the Q192R variant and active drug metabolite levels, change in platelet aggregation or clinical outcomes (cardiovascular death and myocardial infarction) for prasugrel-treated as well as for clopidogrel-treated patients (Mega et al. 2015). A meta-analysis of 13 studies and 16,760 clopidogrel-treated patients in the same publication reinforced the lack of a significant association between Q192R and cardiovascular outcomes in clopidogrel-treated patients (Mega et al. 2015).

Although the CYP2C19 metabolizing enzyme is not involved in ticagrelor pharmacokinetics, there are studies that have evaluated *CYP2C19* genetic polymorphisms and surrogate and clinical outcomes: in stable coronary artery disease patients with *CYP2C19* polymorphism, ticagrelor treatment resulted in lower platelet function than clopidogrel (Tantry et al. 2010); in a sub-study of the PLATO trial, 10,285 acute coronary syndrome patients showed a lower risk of clinical events in the ticagrelor group, compared to clopidogrel. There was no difference in the risk of stent thrombosis and major bleeding compared to clopidogrel in the carriers of the *CYP2C19* loss-of-function allele (Wallentin et al. 2010).

In a recent genome-wide association study in patients of the PLATO trial treated with ticagrelor, the drug pharmacokinetic behaviour was influenced by three genetic loci (*SLCO1B1*, *UGT2B7* and *CYP3A4*). However, the modest genetic effect on ticagrelor plasma levels did not translate into any detectable clinical effect on efficacy or safety during treatment (Varenhorst et al. 2015).

Next Stop: Epigenetics

As already mentioned, a number of candidate gene studies have been designed in the last years to identify possible genetic variants influencing platelet activation and aspirin inhibition. Most of these studies focused on native platelet function and were based on a combination of imputation and genome-wide analysis. With this approach a number of variants, being located in *PEAR1* (rs12041331), *SHH* (rs6943029), *MRVI1* (rs1874445), *ADRA2A* (rs869244), *JMJD1C* (rs2893923) and *GP6* (rs1671152) genes, have been found to be associated to functional-related changes in platelets (Johnson et al. 2010). Interestingly enough, all the variants identified reside in noncoding regions of the genes (either intronic or intragenic), suggesting they may play a role in the expression modulation of these genes rather than influencing their functional properties. In many instances, these noncoding polymorphisms are located in gene regions that are defined as "enhancers" in several pathophysiological conditions but not yet systematically reported in response to antiplatelet therapy variability. Enhancers are important players in gene regulation and could directly link these variants to the changes in gene regulation they might cause in platelets. Three out of the six polymorphisms mentioned above are also introducing a CpG site in the DNA sequence (referred to as "CpG-SNPs"), adding a potential site for methylation to be deposited within the noncoding regions where they are located.

One very interesting example of CpG-SNP is represented by *PEAR1* rs12041331. PEAR1 is highly expressed in platelets and megakaryocytes and participates in both platelet activation and megakaryocyte proliferation (Kauskot et al. 2012, 2013). The minor A-allele of rs12041331 was found to be associated with lower PEAR1 expression in platelets (Faraday et al. 2011). However, in a study involving two independent populations, the minor A-allele was linked to lower platelet function on aspirin but was also found to be a risk factor for cardiovascular events (Lewis et al. 2013). This apparent divergent result still requires further investigations, especially focusing on the molecular mechanisms by which this variant could modulate *PEAR1* expression, eventually through the mediation of DNA methylation. Interestingly, other CpG-SNPs have been recently described for the *CYP2C19* gene (rs12773342, rs12773342, rs12773342) to be associated with coronary heart disease (Ye et al. 2015). However, their mechanism of action still remains elusive.

MicroRNAs (miRNAs) are short noncoding RNAs that work as post-transcriptional regulators of gene expression. Because of their function, miRNAs are present in the circulation via several different mechanisms. miRNAs are cell specific, although some of these molecules are also commonly abundant among some cell types, often when they

contribute to regulate the same pathophysiological processes. One such example is represented by miR-126, abundantly present in both platelets (Landry et al. 2009; Nagalla et al. 2011) and endothelial cells (van Solingen et al. 2009) which has been reported to be altered in various cardiovascular diseases (Corsten et al. 2010; Tijsen et al. 2010; Wang et al. 2010). Plasma levels of this miRNA have been reported to change after treatment with aspirin (de Boer et al. 2013) or aspirin and prasugrel (Zampetaki et al. 2012). Similar observations have also been made for miR-223 (Shi et al. 2013, 2015), while low miR-19b-1-5p expression was linked to aspirin insensitivity in platelets (Kok et al. 2015).

On the other hand, agents like aspirin might play a direct role on the methylation of cells and genes that contribute via effects on platelets to cardiovascular phenotypes. Numerous studies have already shown that aspirin use is associated with a lower incidence of colon neoplasia, metastatic colorectal cancer and death due to colorectal cancer (Algra and Rothwell 2012; Bosetti et al. 2012; Maxwell 2012; Thun et al. 2012). However, few studies are now available on more vascular disease-related cell types such as endothelial cells (Chang et al. 2013). It is hoped that future epigenomic studies in the field might better unravel this possible dual epigenetic-mediated influence of antiplatelet therapy.

Conclusions

Over the last two decades, a number of studies on the possible genomic regulation of common "polygenic" ischemic cardio- and cerebrovascular diseases have been published. These studies have identified numerous DNA variants as disease or antiplatelet drug susceptibility markers. Some of these DNA variants confer a variable increase in risk for disease, while loss-of-function variants in the hepatic *CYP2C19* system have been reported to be the predominant genetic mediators of clopidogrel and other thienopyridine drug antiplatelet response. However, the overall available data are still somewhat contradictory and do not yet allow a clear role for pharmacogenomics in the context of providing effective, reliable, personalized antiplatelet therapy. However, it is anticipated that in the near future, the relative role of additional rare polymorphisms, structural variants and tissue-specific epigenetic features of the human genome (including DNA methylation or acetylation, histone modifications and miRNA post-translational regulation of gene expression) as significant contributors to cardio- and cerebrovascular disease pathogenesis and drug response will hopefully be defined. Future findings will be useful to radically change our practice of cardio- and cerebrovascular medicine.

Pharmacogenomics of Antiplatelet Drugs: Key Concepts

- Pharmacogenomics has been implicated in the individual response variability to antiplatelet drugs in patients with ischemic vascular disease, but no clear conclusions nor definite guidelines are available on the clinical advantages of pharmacogenetic testing before prescribing antiplatelet drugs.
- Residual on-treatment platelet response to aspirin measured by platelet function tests other than TxB2 measurement significantly correlated with the lack of clinical response in patients with cardiovascular disease and with an excess in the occurrence of future cardiovascular events compared to "responder" patients.
- Candidate gene studies have not provided consistent evidence for a critical gene-aspirin response relationship: this is possibly due to inadequate assessment of phenotype or to the fact that the contribution of each single gene variant to the overall phenotypic variance was too low and/or the population size too small.
- Clopidogrel is absorbed as a prodrug and converted to its active thiol metabolite by two sequential oxidative steps through the hepatic cytochrome (CYP) P450 enzymes. Among the CYP isoenzymes having a relevant metabolic role, the polymorphically expressed *CYP2C19* affects both metabolic steps of clopidogrel active metabolite generation and therefore plays a dominant role in this process.
- The results of many studies (and meta-analyses) on the association between *CYP2C19* loss-of-function alleles and clinical efficacy of clopidogrel were discordant; in three large trials (GRAVITAS, ARCTIC and TRILOGY-ACS), bedside-tested platelet reactivity did not result in any clinical benefit.
- In 2010, the US Food and Drug Administration added a boxed warning to the clopidogrel label, with an alert to clinicians on the reduced effectiveness of clopidogrel in patients who are poor metabolizers of the drug, leaving however the decision to use genetic tests to the decision of physicians, a recommendation currently not evidence based.
- Intensified antiplatelet therapy (peri-procedural GPIbIIa inhibitor infusions, repeated loading dose or increased maintaining dose of clopidogrel or use of prasugrel) tailored on the basis of individual platelet reactivity tests in prospective studies was

(continued)

associated with a significant reduction in cardiovascular mortality, stent thrombosis and myocardial infarction, without any difference in major bleeding events. However, the net clinical benefit of the intensified treatment versus the standard one significantly depended on the risk of stent thrombosis with a standard dose of clopidogrel.
- Recent prospective proof-of-concept trials, in which patients undergoing percutaneous coronary intervention were randomized to either point-of-care genotyping and subsequent personalized treatment or to standardized clopidogrel, showed that the genetic clopidogrel profile was a good marker of platelet function response on clopidogrel but was not related to clinical outcome, suggesting that the genetic added little to the pharmacodynamic information used in the study to adjust antiplatelet therapy. These findings support the conclusion that there might still be room for optimization of antiplatelet therapy by genotyping.
- The relative role of additional rare polymorphisms, structural variants and tissue-specific epigenetic features of the human genome will hopefully be defined in the near future as significant contributors to the pathogenesis of cardio- and cerebrovascular disease and of individual patient's response to antiplatelet drugs.

Take Home Messages

- Pharmacogenomics has been implicated in the individual response variability to antiplatelet drugs in patients with ischemic vascular disease, but no clear conclusions nor definite guidelines are presently available on the clinical advantages of pharmacogenetic testing before prescribing antiplatelet drugs.
- In the meantime individual variability can be tested by residual on-treatment functional platelet response to aspirin or other antiplatelet drugs.
- Intensified antiplatelet therapy (peri-procedural GPIbIIa inhibitor infusions, repeated loading dose or increased maintaining dose of clopidogrel or use of prasugrel) can be tailored on the basis of individual platelet reactivity tests to obtain a significant reduction in cardiovascular mortality, stent thrombosis and myocardial infarction, without minor differences in major bleeding events.

- The relative role of additional rare polymorphisms, structural variants and tissue-specific epigenetic features of the human genome will hopefully be defined in the near future as significant contributors to the pathogenesis of cardio- and cerebrovascular disease and of individual patient's response to antiplatelet drugs.

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Targeting Intraplatelet Signaling Pathways as Potential Antithrombotic Strategy

Patrick Andre

Abstract

Platelets assume two main functions that lead to opposite outcomes. In one instance, they support hemostatic plug formation and prevent bleeding at sites of vascular breach. Alternatively, their uncontrolled recruitment at sites of plaque erosion/rupture in arteries can lead to ischemic events and death. Antiplatelet agents have been developed to successfully treat or prevent arterial thrombotic events, but their use is inevitably associated with bleeding complications. These agents target platelet receptors (i.e., $P2Y_{12}$, the ADP receptor mediating aggregation and thrombus stability; $\alpha_{IIb}\beta_3$, the fibrinogen and vWF receptor mediating platelet aggregation/thrombus growth; PAR1, the high-affinity platelet thrombin receptor) or the cyclooxygenase 1 (COX1) enzyme that promotes TxA_2 synthesis (another platelet-derived endogenous agonist of platelet aggregation). Evidences have emerged showing that these pathways are complementary as combinations of drugs provide additive protection. Signaling pathways downstream of the glycoprotein receptors have been well characterized and indicate that inhibition of effectors (specific to a pathway or ubiquitous) could provide significant protection from thrombosis. In this chapter, we will review the biology associated with genetic and pharmacologic modulation of platelet effectors and report their effects on thrombosis and primary hemostasis.

Platelet Signaling

Signaling Downstream GPVI-FcRγ and GPIbα

Glycoprotein VI (GPVI), glycoprotein Ib-IX-V (GPIb), and integrins share a common signaling pathway that relies on the engagement of the Src family kinases, the ITAM domain of FcRγ, and the phosphoinositide 3-kinases (PI3K) (Fig. 1). Platelet adhesion to collagen requires GPVI-mediated activation, and deletion or depletion (via use of an antibody) of GPVI in mice is associated with protection from thrombosis and a moderate impairment of the hemostatic response (for review, see Nieswandt and Watson (2003)). GPVI is comprised of two Ig domains followed by a mucin-like region linked to a transmembrane domain possessing an arginine group that allows the association with FcRγ. The FcRγ-chain is a disulfide-linked homodimer with an ITAM sequence in which two tyrosine residues play a critical role in the collagen-FcRγ signaling pathway. The GPVI-FcRγ-chain complex binds to the glycine-proline-hydroxyproline sequence of fibrillar collagen. As both Lyn and Fyn are constitutively bound to the cytoplasmic domain of GPVI via their SH3 domains, cross-linking of GPVI by collagen occurs and brings Fyn and Lyn to the ITAM region in the FcRγ-chain (not represented in this illustration). The Src kinases mediate the phosphorylation of the two YXXL conserved motifs of FcRγ-ITAM. This phosphorylation offers docking sites for the SH2 domains of the spleen tyrosine kinase (Syk), leading to the activation of its kinase function. Syk autophosphorylation happens, allowing it to dissociate from the ITAM domain and to recruit key signaling enzymes. It is believed that such

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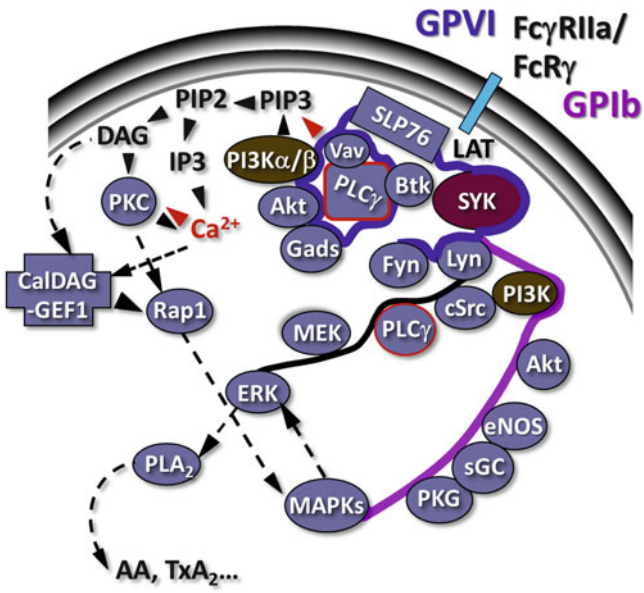

Fig. 1 Platelet signaling pathways downstream ITAM-coupled receptors

activity is driven by its ability to engage the PI3K-Akt-eNOX-sGC-PKG pathway (Li et al. (2010); purple line). The importance of Syk in collagen-dependent platelet activation is illustrated by the fact that neither FcRγ$^{-/-}$ nor Syk$^{-/-}$ platelets respond to collagen-related peptide (CRP) or collagen (Poole et al. 1997). It should be noted that the murine phenotypes associated with these two deficiencies differ substantially as Syk (which is also part of the CLEC2 signaling pathway) deficiency also impacts vasculogenesis, being associated with a severe bleeding diathesis, while FcRγ$^{-/-}$ animals do not show prolonged bleeding time (Poole et al. 1997). Activation of the Syk SH2 domains is at the origin of a signaling cascade involving adapters and effector enzymes (blue line). Activated Syk phosphorylates the transmembrane adapter linker for activated T-cells (LAT) and SLP-76. A complex assembles, comprised of Bruton's tyrosine kinase (Btk), Gads, and phospholipase C (PLC)-γ2. This complex acts as a positive autocrine loop to further enhance the activation PLC-γ2 (Watson et al. 2005). PLC-γ2 hydrolyzes PtdIns (4,5)P_2, cleaving the bond between the glycerol and phosphate moieties, generating the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 is known to release cytosolic calcium from internal stores, while DAG activates protein kinase C (PKC) isoforms α and β that mediate secretion of α-granules, aggregate formation and phosphatidyl serine (PS) expression on the platelet surface (Gilio et al. 2010). The small GTPase Rap1b plays a key role in several of these processes, as it is directly activated by PKC and the calcium-sensing guanine nucleotide exchange factor, CalDAG-GEFI. Activation of PLC-γ2 ultimately leads to activation of PLA_2, release of arachidonic acid followed by the synthesis of thromboxane A_2 (TxA_2), release of granule contents, and activation of $\alpha_{IIb}\beta_3$.

Platelet signaling pathways downstream of GPVI and GPIbα engagement by collagen (CRP/convulxin) and vWF, respectively, have been extensively studied and are well characterized (see above). Figure 2 highlights the defects in enzyme phosphorylation, α- and dense-granule secretion, and $\alpha_{IIb}\beta_3$ activation/aggregation associated with deficiency in key signaling enzymes/effectors involved in the response to collagen/CRP stimulation in mice. The enzymes are shown according to their predicted order in the signaling pathway. Figure 2 also presents the impact of the genetic manipulation (or pharmacological one) on primary hemostasis and reports whether deficiency provides a biological impairment beyond platelet function.

The GPIb-V-IX complex encompasses four subunits. GPIbα and β are disulfide-linked and non-covalently associated with GPV and GPIX. It is commonly believed that the Ibα subunit carries most of the activities as it possesses the binding site for vWF, thrombin, P-selectin, Mac1, and FXI [for review, see Lopez and Dong (1997); Berndt et al. (2001)]. GPIbα interaction with vWF under arterial shear rates facilitates the recruitment of platelets at the vessel wall. This process is initiated by at least two intraplatelet signaling pathways. The cytoplasmic domain of GPIbα associates with Lyn and PI3K. Lyn activates PI3K which in turn leads to the activation of Akt (Angelillo-Scherrer et al. 2005). This triggers the activation of the eNOS/sGC/PKG (cGMP-dependent protein kinase), mitogen-activated protein kinase (p38 MAPK), and extracellular signal-regulated kinase (ERK). GPIbα is also associated with ITAM receptors and, by consequence, shares this signaling pathway with GPVI (Gardiner et al. 2010). Indeed, tyrosine phosphorylation following vWF binding to GPIbα requires the recruitment of Src and Lyn, Lyn activation, followed sequentially by the phosphorylation of Syk, SLP-76, PI3-kinase, Btk, PLCγ2, and PKC, ultimately resulting in elevation of cytosolic Ca^{++} and $\alpha_{IIb}\beta_3$ activation (see Fig. 1). GPIbα also binds thrombin with high affinity, but until recently the functional importance of this interaction was controversial (De Candia et al. 2001; Ramakrishnan et al. 2001). A recent study by Du and colleagues indicates, however, that low concentrations of thrombin induce cooperative signals between GPIbα and PARs (Estevez et al. 2015).

Signaling Downstream Protease-Activated Receptors (PARs) and GPCRs (Fig. 3)

Thrombin activates platelets by cleaving protease-activated receptors (PAR)1 and PAR4 in humans (PAR3 and PAR4 in mice), revealing a new tethered ligand that binds to and activates the receptor. The signaling pathways initiated by the cleavage of PAR1 (high-affinity receptor) and PAR4 (low-affinity receptor) involve heterotrimeric G proteins

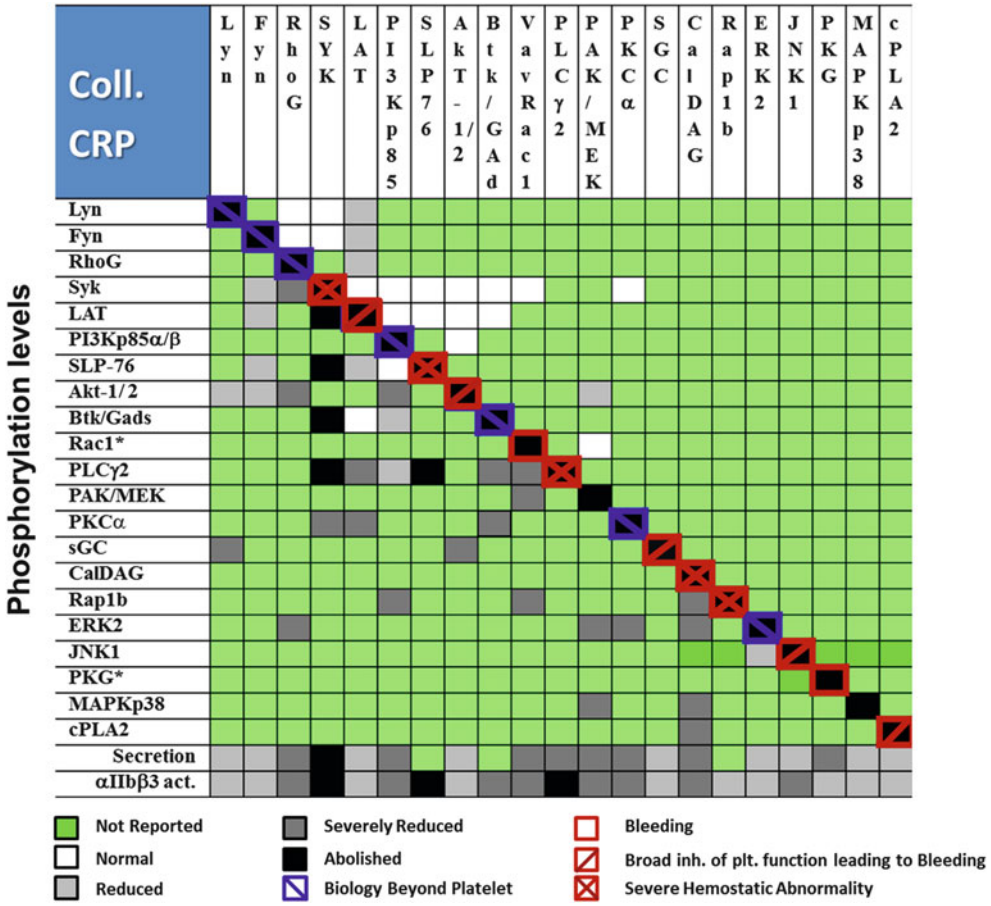

Fig. 2 Impact of genetic deficiency of pharmacological modulation of key platelet effectors on phosphorylation levels, platelet activation, and secretion

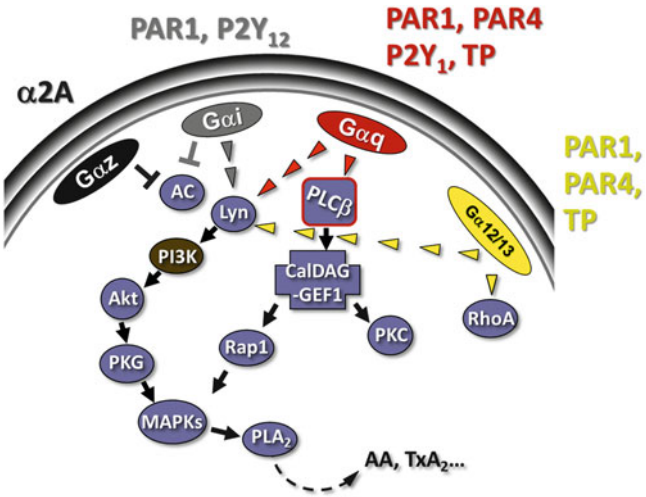

Fig. 3 Platelet signaling pathways downstream PARs and other GPCR receptors

[$G_{\alpha 12/13}$,$G_{\alpha q}$, and $G_{\alpha i/o}$ for PAR1 (Ayoub et al. 2010); $G_{\alpha 12/13}$ and $G_{\alpha q}$ for PAR4] (for review see Russo et al. (2009)). Interestingly, proteases cleave PARs at specific and potentially divergent sites within the extracellular domain, activating the canonical pathways of G proteins and β-arrestin and also different signaling cascade (commonly referred to as biased signaling). It should also be noted that subtle differences exist in signaling pathways between the two receptors as a result of the release of secondary mediators of platelet aggregation (e.g., ADP secretion) and the fact that PAR1 directly activates $G_{\alpha i}$ (Holinstat et al. 2006). In brief, $G_{\alpha q}$ and $G_{\alpha i}$ signaling involving the phospholipase Cβ-CalDAG-GEFI-PKC pathway and the PI3K-Akt-PKG axis or PI3K-Rasa3-Rap1 axis, respectively, converges to induce secretion of the α-granules and dense granules, Rap1 and $\alpha_{IIb}\beta_3$ activation, and platelet aggregation. $G_{\alpha 12/13}$-dependent activation of p115-RhoGEF-RhoA also occurs and leads to platelet shape change. It is believed that thrombin induces activation of Rho and Rac, triggering activation of p21-activated kinase (PAK) at focal adhesion and lamellipodia regions in platelets. PAK plays a key role in platelet filopodia and lamellipodia formation downstream of RhoA, Cdc42, and Rac1, associating with Rac and regulatory proteins such as GEFH1, GIT, and PIX (which also serve as substrate) (Sato et al. 2008). PAK notably

phosphorylates LIMK1 (together with ROCK) resulting in the inactivation of cofilin and, by consequence, in an increase in actin polymerization (Bernard 2007), and activation of Merlin, MAP kinase, and Akt and the release of calcium (Aslan et al. 2013). Finally, Pyk2, a non-receptor tyrosine kinase, interacts with the actin cytoskeleton and gets activated upon thrombin stimulation (and also by ADP and U46619). Interestingly, the $G_{\alpha 12/13}$ signaling cascade is also common to other GPCR receptors such as the TxA_2/PGH_2/isoprostane receptor (TP, which couples with $G_{\alpha q}$ and $G_{\alpha 12/13}$) and the ADP receptors $P2Y_{12}$ (coupled with $G_{\alpha i}$-AC) and $P2Y_1$ (coupled with $G_{\alpha q}$).

$\alpha_{IIb}\beta_3$ Outside-In Signaling

$\alpha_{IIb}\beta_3$ engagement by its ligand promotes platelet adhesion and aggregation and initiates signaling pathways in platelets that lead to cytoskeletal reorganization, spreading, secretion, and clot retraction. It induces the assembly of signaling molecules from which the outside-in signaling originates. c-Src plays a key role in this process via a direct interaction with the β_3 cytoplasmic domain of the receptor (Arias-Salgado et al. 2003; Ablooglu et al. 2009; Wu et al. 2015) and initiation of the PI3K pathway (O'Brien et al. 2012). Its activation follows the coupling of $G_{\alpha 13}$ to the cytoplasmic tail of β_3 (Gong et al. 2010). $\beta_3(\Delta 760–762)$ knock-in mice show reduced tyrosine phosphorylation of c-Src and a defective outside-in signaling pathway in platelets. This platelet function defect is associated with thromboprotection but also prolongation of bleeding time, although the heterozygous animals only displayed moderate prolongation (Ablooglu et al. 2009). Other protein-tyrosine kinases have key functions in mediating outside-in signaling, including FAK (focal adhesion kinase), ILK, Syk, and Src family kinase (SFK). The inhibition of RhoA by c-Src and activation of PI3K mediate platelet spreading on ligands, while PI3K and Syk signaling promotes platelet secretion. Phosphorylation of β3 is also known to control cytoskeletal changes that directly impact platelet function [for review see Estevez et al. (2015)].

Effectors of Platelet Function not Involved in Primary Hemostasis

Lyn

Genetic Modulation and Pathophysiology

Lyn is a non-receptor protein-tyrosine kinase, which belongs to the Src family of tyrosine kinases among Src, Fyn, Hck, Yes, Lck, and Fgr. Lyn associates with the GPVI-FcRγ complex where it sequentially plays stimulatory and inhibitory roles. On the one hand, through its constitutive interaction with the cytoplasmic domain of GPVI, it contributes to the release of platelet granules via the PI3K pathway (Fig. 1, purple line). On the other hand, it promotes the phosphorylation of PECAM-1, which leads to the recruitment of SHP-2 phosphatase (Li et al. 2010; Ming et al. 2011). A similar dichotomy has been reported downstream of the thrombin receptor (Cho et al. 2002; Li et al. 2010). Interestingly, Lyn also plays an important role in the activation of $\alpha_{IIb}\beta_3$ (inside-out signaling) downstream of GPIbα engagement by vWF, a signaling event believed to be mediated by the cGMP signaling pathway, but it does not seem to contribute to outside-in signaling (downstream of $\alpha_{IIb}\beta_3$ engagement by fibrinogen) (Yin et al. 2008). Overall, Lyn deficiency does not lead to bleeding complications but is only associated with moderate inhibition of thrombosis.

Pharmacological Modulation

Based on these findings, one could speculate that monotherapy targeting Lyn would have limited effect on thrombosis. It is possible, however, that modulation of Lyn could synergize with other modulators of platelet function. Development of a selective Lyn inhibitor for the prevention or treatment of thrombosis will necessitate an evaluation of its activity in presence of other platelet antagonists for establishment of target engagement levels and therapeutic windows. This is particularly important as Lyn is also known to control cell cycle, notably in B-cells (Scheuermann et al. 1994), and it works across different cellular signal transduction pathways. In addition, high levels of selectivity would be required against other kinases, notably from the Src family, to prevent effects beyond thrombosis [e.g., see dasatinib mechanism of action and effects in Inge et al. (2013)]. Thus, therapeutic windows would have to be first established in translational preclinical species prior to committing to a full drug discovery program in the thrombosis arena.

Fyn

Genetic Modulation and Pathophysiology

In platelets, Fyn plays a key role in Akt phosphorylation downstream of GPVI and GPIbα engagement (Yin et al. 2008; Kim et al. 2009). It is also involved in outside-in signaling (Kim and Kunapuli 2011). Lack of Fyn is compatible with development and not associated with overt bleeding, although a tendency to rebleed was noticed (Reddy et al. 2008).

Pharmacological Modulation

Fyn kinase inhibitors have been developed but, so far, for non-thrombotic indications. Indeed, Fyn is ubiquitously

expressed across the human body and has been shown to also participate in T-cell signaling, cell growth and survival, adhesion and motility, axon guidance and synaptic function, and myelination [for review see Elias and Ditzel (2015)]. Fyn has been involved in cancer pathogenesis through a direct participation in mechanisms leading to cell growth and death, transformation, and motility, in drug-resistance mechanisms, and in signal transduction pathways in the nervous system. Modulation of Fyn is currently being studied as a potential therapy for the development of Alzheimer disease (Nygaard et al. 2014). As an example, AZD0530, a Fyn kinase inhibitor, showed ability to rescue spatial memory deficits and synaptic depletion (Kaufman et al. 2015). Hence, due to ubiquitous function and distribution and a relatively limited participation to platelet biology (i.e., aggregation), this target does not seem to have triggered development of a specific inhibitor for thrombosis indication.

Rho

Genetic Modulation and Pathophysiology

Rho GTPase such as RhoA, Rac1, and Cdc42 possesses roles in platelet function, cytoskeletal rearrangement, as well as platelet formation (Aslan and McCarty 2013; Pleines et al. 2013). They bind GTP to induce the activation of downstream effectors in a manner relatively similar to that of G_{α} subunits (see Stefanini et al. 2017). Of those GTPases, RhoG regulates microtubule rearrangements, filopodia and lamellipodia formation, spreading and granule trafficking across several cell types (B-cells, T-cells, endothelial cells, platelets (Goggs et al. 2015). $RhoG^{-/-}$ mice have been shown to possess increased levels of IgG1 and two antibodies and a mild defect in B- and T-cell activities (Vigorito et al. 2004). Use of $RhoG^{-/-}$ platelets highlighted a key participation to $\alpha_{IIb}\beta_3$ activation and platelet aggregation upon platelet stimulation by CRP and thrombin, a process initially proposed to be driven by Src and Syk kinases (Goggs et al. 2013). Further work by the group of Dr. Kunapuli demonstrated that RhoG acts upstream of Syk, Akt, and ERK in the GPVI signaling pathway and significantly contributes to thrombus formation in vivo. Interestingly, granule secretion is differentially impaired in $RhoG^{-/-}$ platelets, with normal response observed following thrombin stimulation but not when CRP is the agonist. Such difference may account for the fact that RhoG-deficient animals have defective thrombus formation while presenting normal primary hemostasis (Goggs et al. 2013). This phenotype resembles that of $FcR\gamma^{-/-}$ mice, which too impact Syk phosphorylation, affect thrombosis but are not involved in cessation of bleeding. Since knockdown of RhoG is associated with a relatively mild bleeding phenotype (as opposed to other Rho GTPases), it could constitute a good antiplatelet target.

Pharmacological Modulation

Despite preclinical data indicating that RhoG could constitute a safe antithrombotic target, there is no evidence that its modulation is actively being pursued for thrombotic indications. Rho-kinase inhibitors are being studied for other indications, such as modulation of ocular hypertension. Preclinically, fasudil has been shown to cause increased cerebral blood flow and protect against cerebral death in an endothelium-derived NO manner (Rikitake et al. 2005; Koumura et al. 2011).

PI3K

Genetic Modulation and Pathophysiology

Four class I phosphoinositide 3-kinases (PI3K) have been reported in platelets. PI3Kβ mediates platelet signaling downstream of GPVI engagement by collagen, plays a role in outside-in integrin signaling, and participates in signaling downstream GPCRs (Jackson et al. 2005; Canobbio et al. 2009; Kim et al. 2009; Martin et al. 2010a, b). Signaling downstream of GPVI has been particularly well studied. The p85α subunit coprecipitates with FcRγ and the LAT adaptor protein (Gibbins et al. 1998) and then brings the p110 subunit to the membrane where it is responsible for the generation of the second messengers phosphoinositide (PI) (3,4)P2 and (3,4,5)P3. PI3Kα is key to the GPVI signaling pathway, and its deficiency affects collagen and CRP signaling and aggregation but not that induced by ADP or thrombin (Watanabe et al. 2003). Finally, PI3Kγ is also contributing to ADP-induced platelet aggregation and plays a role in thrombosis (Hirsch et al. 2001; Canobbio et al. 2009).

Pharmacological Modulation

Interestingly, while deficiency in p85α and p85β has been associated with protection from thrombosis at no cost on bleeding, data from experimental studies evaluating activity of pharmacological inhibitors are somehow more controversial. As an example, TGX-221, a PI3Kβ inhibitor that blocks the Rap1b/Akt pathway downstream of ADP-induced activation (see Jackson et al. 2005; Gilio et al. 2009), prolonged bleeding time, a phenomenon likely explained by the fact that PI3Kβ plays a role in collagen, ADP, and also thrombin-induced signaling. Similar findings were also observed by Bird and colleagues with a PI3Kβ antagonist (Bird et al. 2011). A potential explanation could come from the fact that PI3K is ubiquitously expressed and that only highly selective inhibitors should be utilized in and across preclinical species. The requirement for highly selective antagonist is further exemplified by the fact that the different isoforms

have key roles in many processes, such as oncogenesis [α, (Stephens et al. 2005), δ (Sanford et al. 2015)], adaptive immunity, and inflammation [γ, (Ruckle et al. 2006), (δ, (Ali et al. 2004)]. PI3K inhibitors have been developed and tested in human. AZD6482 (ClinicalTrials.gov Identifier: NCT00688714) was notably evaluated vs clopidogrel on bleeding time when taken together with low-dose aspirin (ClinicalTrials.gov Identifier: NCT00853450). Another drug, TG100-115, underwent Phase I/II studies and was designed for heart attack treated with angioplasty (ClinicalTrials.gov Identifier: NCT00103350); however the different findings have not been released yet.

Btk

Genetic Modulation and Pathophysiology

Bruton's family kinases (Btk) are non-receptor tyrosine kinases involved in a multitude of signaling pathways, amplifying signals coming from the cell membrane across a wide variety of cells. They participate in signal transduction downstream of GPCRs, integrins, RTKs, cytokine, and death receptors and interact with nearly all non-receptor tyrosine kinases. They have been reported to activate/phosphorylate PLCγ, PKC, Rho, WASP, actin, BCL-XL, Akt, JNK, Stat3, TFII-I, and NFκB [for review see Qiu and Kung (2000)]. Btk is involved in cell growth, differentiation, and development. As an example, its deficiency prevents proliferation of B-cells upon antigen stimulation, and mutations in human have been linked to immunodeficiency disease, X-linked agammaglobulinemia (XLA), and apoptosis. In platelets, Btk (a member of the Tec family) regulates PLCγ2 activity following engagement of GPVI by collagen and CRP. Btk is regulated by Src kinases, phosphorylated in response to collagen and thrombin stimulation and recruited to the plasma membrane via an interaction with PIP3. Platelets from XLA patients have a defect in activation induced by collagen and CRP (Quek et al. 1998) that can be compensated by high concentrations of platelet agonist.

Pharmacological Modulation

Several compounds targeting Btk (e.g., ibrutinib, acalabrutinib, PCI-32765, HM-71224, CC-292, and ONO-4059) have entered clinical evaluation for the treatment of hematologic malignancies and autoimmune disorders. Interestingly, some bleeding symptoms have been observed in chronic lymphocytic leukemia and mantle cell lymphoma patients treated with ibrutinib, an observation in conflict with preclinical results (Rigg et al. 2015). Furthermore, antithrombotic effects could be observed preclinically with ibrutinib but not with acalabrutinib (Byrd et al. 2016). Thus, selectivity profile of Btk inhibitors will be an area of concern as well as safety and determination of effective thrombosis target engagement levels for long-term chronic use.

PAK

Genetic Modulation and Pathophysiology

PAK (p21-activated kinases) are serine/threonine protein kinases Rho GTPase effectors promoting cytoskeletal rearrangement. PAK2 is activated downstream of PAR receptor engagement (Arias-Romero and Chernoff 2008), where they support activation of the MEK/ERK pathway (Aslan et al. 2013). Nevertheless, there have been no reports on potential involvement in primary hemostasis in preclinical species. Although there is only sparse information relative to the phenotype associated with modulation or deficiency of PAKs, it should be emphasized that mast cell deficiency in PAK1 is associated with decreased allergen-induced degranulation.

Pharmacological Modulation

PAK4 inhibitor (PF-03758309) is currently being tested in human in patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCT00932126). There are no reports on the development of PAK2 inhibitors. Based on its broad involvement in multiple cell signaling pathways, it is likely that highly selective inhibitors would also have to be developed in order to limit the biology to the platelet function.

LIMK1

Genetic Modulation and Pathophysiology

LIMK1 (LIM kinase 1) is expressed in platelets and is activated via phosphorylation of threonine residues (notably through p21-activated kinases) upon platelet activation and is believed to facilitate actin polymerization. Deficiency in LIMK1 is associated with protection from thrombosis without significant effect on primary hemostasis (Estevez et al. 2013). LIMK1 participation to thrombosis is dual as it promotes a GPIbα-dependent TxA_2 synthesis and also integrates responses to low-dose thrombin between GPIb and PAR signaling pathways (Estevez et al. 2015).

Pharmacological Modulation

There are no known modulators of LIMK1 in development.

PKC

Genetic Modulation and Pathophysiology

Human and murine platelets express the PKC isoforms α, β, δ, and θ. PKCα is considered key to platelet degranulation and aggregation in response to collagen and thrombin (human), while PKCβ (mice) supports outside-in signaling (Buensuceso et al. 2005; Konopatskaya et al. 2009; Gilio et al. 2010). Redundancy in isoforms certainly accounts for the lack of bleeding prolongation in PKCα-deficient mice. Perfusion of blood over collagen revealed that while PKCα and β actively contribute to the thrombotic process, the other two isoforms (δ and θ) may act as endogenous inhibitors (Crosby and Poole 2003; Chari et al. 2009). PKCs have broad activities in multiple cell types, being involved in proliferation, differentiation, adhesion, motility, and apoptosis. PKCα expression levels and activity are often increased in tumor tissues [currently the focus of several cancer therapies (Sliva 2004)]. PKCα is also involved in T-cell biology (Pfeifhofer et al. 2006; von Essen et al. 2006) and insulin homeostasis (Leitges et al. 2002), and its modulation has been shown to affect heart failure (Braz et al. 2004).

Pharmacological Modulation

Several PKC inhibitors are currently being tested in clinical studies, notably in the cardiovascular/cardiometabolic area. LY333531, a PKCβ inhibitor has been studied in diabetic patients, notably for reduction in vision loss associated with diabetic retinopathy (ClinicalTrials.gov identifier: NCT00604383). Safety and efficacy studies of KAI-9803 have also been performed in subjects with ST-elevation myocardial infarction (PROTECTION AMI, ClinicalTrials.gov Identifier: NCT00785954), while others are being investigated in multiple transplantation and oncology settings (e.g., AEB071). There are no published reports indicating that such compounds are being developed for thrombosis indications.

Vav1/3

Genetic Modulation and Pathophysiology

Vav1- and Vav3-deficient platelets have a defect in GPVI signaling in response to collagen stimulation, upstream of PLCγ2 (Pearce et al. 2004). Interestingly, Vav has been associated with oxidized LDL-mediated platelet activation, linking thrombosis and hyperlipidemia (Chen et al. 2011).

Pharmacological Modulation

There are no known modulators of Vav1/3 in development.

PLD1

Genetic Modulation and Pathophysiology

Mice deficient in phospholipase D1 have been generated and displayed a defect in GPIbα signaling pathway leading to inhibition of arterial thrombosis at no cost on bleeding. The platelets have an overall impairment of $\alpha_{IIb}\beta_3$ activation in response to most major platelet agonist (Elvers et al. 2010). PLD1 is also involved in other pathologies and has been linked to Alzheimer's disease and tumor angiogenesis and metastasis (Jin et al. 2007; Chen et al. 2012)

Pharmacological Modulation

A PLD1 inhibitor, 5-fluoro-2-indolyl des-chlorohalopemide, has been shown to block the enzyme activity and interfere with granule release and inside-out signaling. It provided protection in an animal model of ischemic stroke with no spontaneous bleeding or prolongation of tail bleeding time, confirming the phenotype of the deficient mice (Stegner et al. 2013).

ERK/p38MAPK

Genetic Modulation and Pathophysiology

Extracellular signal-regulated kinase (ERK) belongs to the mitogen-activated protein (MAP) kinase family involved in cell signaling and adhesion. Genetic inactivation of ERK2 results in early embryonic lethality (Aouadi et al. 2006). In addition, cardiac and craniofacial defects as well as developmental delay and mental retardation have been reported in patients with mutations in the ERK/MAPK pathway (see Samuels et al. 2008). P38 MAPK has also been reported to play a role in models of lung metastasis (Matsuo et al. 2006). In platelets, ERK2 activation downstream of collagen stimulation is Rap1 dependent (Stefanini et al. 2009) and requires TxA_2/TP (mostly G_q) and ADP/$P2Y_{12}$ (G_i signaling) signaling (Roger et al. 2004). Experiments performed under flow conditions revealed that ERK2 and p38 MAPK have complementary roles in adhesion to collagen (Mazharian et al. 2005), that participation of ERK2 requires engagement of GPIbα by vWF and the $P2X_1$ ion channel (via PKC/MEK1/2), while that of p38 involves integrin $\alpha_2\beta_1$. ERK2 is also activated in response to thrombin cleavage of PAR1/4, a phenomenon also attributed to PKC and MEK1/2 (Aharonovitz and Granot 1996). Similarly, thrombosis was delayed in a ferric chloride thrombosis model applied to the carotid artery of $p38MAPK^{+/-}$ mice, a phenotype notably attributed to a defect in $\alpha_{IIb}\beta_3$ activation mediated by inhibition of the TP pathway, likely through a defect in cPLA2 phosphorylation (Borsch-Haubold et al. 1998).

Pharmacological Modulation

Of note, use of a selective MEK1/2 inhibitor in wild-type and transgenic mice overexpressing $P2X_1$ delayed time to occlusion in injured arteries through inhibition of the vWF-GPIbα pathway (Oury et al. 2006). Losmapimod (a p38 MAPK inhibitor, GSK) benefits are currently being tested in a Phase III study in patients with acute coronary syndrome. The hypothesis is that inhibition of p38 MAPK will stabilize atherosclerotic plaques and prevent subsequent thrombosis leading to ischemia. VX-702 (Vertex) has shown an ability to decrease platelet storage lesion in vitro (Wagner et al. 2015) as well as platelet aggregation (Kuliopulos et al. 2004) and has been tested in the setting of rheumatoid arthritis as well as patients with acute coronary syndrome undergoing PCI. p38 MAPK inhibitors are also being developed for indications as diverse as COPD, asthma, rheumatoid arthritis, Alzheimer's, and oncology (Clinicaltrials.gov).

Effectors Involved in Thrombosis and Primary Hemostasis

LAT

Genetic Modulation and Pathophysiology

LAT, or Linker for activation of T-cell, is expressed in T-cells, NK cells, macrophages, mast cells, and platelets. It is found in glycolipid-enriched domains (Zhang et al. 1998a, b) where it associates with several proteins involved in T-cell receptor signaling (Zhang et al. 1998a, b). LAT deficiency in mice is characterized by an absence of mature peripheral T-cells (Zhang et al. 1999). LAT is tyrosine phosphorylated upon collagen stimulation and FcγRIIA cross-linking in platelets (Pasquet et al. 1999). It associates with p85PI3K and the FcRγ-chain but also with SLP-76, PLCγ2, Cbl, and Grb2 (Asazuma et al. 2000). The main consequence of platelet LAT deficiency is a reduced phosphorylation and activation of PLCγ2 that leads to a defect in platelet secretion and aggregation following activation by collagen. LAT deficiency is accompanied by a mild increase in bleeding time similar to that observed in FcRγ-deficient mice and a strong protection from thrombosis (at least similar to that conferred by FcRγ and PLCγ2 deficiency) in models exposing fibrillar collagen to the circulating whole blood (Kalia et al. 2008).

Pharmacological Modulation

There are no known inhibitors of LAT in development for the treatment/prevention of thrombosis.

Akt(s)

Genetic Modulation and Pathophysiology

Akt1 and 2 are serine/threonine kinases found in human and murine platelets. While Yin et al. found that Akt-$1^{-/-}$ mice have a defect in their platelet response to collagen and thrombin accompanied by a decrease in granule release and significantly increased tail bleeding times (Chen et al. 2004; Yin et al. 2008), some have argued that Akt2 is the predominant functional isoform as mice lacking Akt2 present a defect in platelet secretion, aggregation, and thrombus formation. This defect has been attributed to an impairment in the signaling pathway downstream of Gq-coupled receptors (PARs and TP) resulting in reduced secretion of α- and dense granules but without a prolongation of bleeding time values (Woulfe et al. 2004). Interestingly, Akt1 has been linked to organismal growth (Cho et al. 2001a, b), while high expression levels of Akt2 in pancreatic B-cells and brown fat have been related to its involvement in glucose metabolism (Akt$2^{-/-}$ mice are hyperglycemic and hyperinsulinemic and present defects in insulin responses (Cho et al. 2001a, b; Garofalo et al. 2003)).

Pharmacological Modulation

Akt inhibitors are being developed for indications as diverse as relapsed lymphoma, diffuse large B-cell lymphoma, and multiple myeloma. Thus, ubiquitous functions are likely to hamper the development of Akt inhibitors for the treatment or prevention of thrombosis.

Rac

Genetic Modulation and Pathophysiology

Rac GTPase 1 (Rac1) is detected in platelets and its activation following GPCR stimulation or $\alpha_{IIb}\beta_3$ engagement has been well documented (Hartwig et al. 1995; Azim et al. 2000; Soulet et al. 2001). Rac1 deficiency is associated with a severe defect in B-cell development (Walmsley et al. 2003), defective GPVI-mediated platelet activation and aggregation due to impaired PLCγ2 activation (Pleines et al. 2009), and a defect in granule secretion (Akbar et al. 2007). Rac1 was also found to mediate platelet procoagulant response to low-dose agonists (Delaney et al. 2014) and to be at the juncture of cooperative signaling pathways downstream of thrombin activation of GPIbα and PARs (Estevez et al. 2015). According to its platelet pleiotropic effects, it is not a surprise to observe prolonged bleeding time in knockout mice (Akbar et al. 2007).

Pharmacological Modulation

There are no reports related to the development of Rac1 inhibitors for thrombosis or cardiovascular indications.

RhoA

Genetic Modulation and Pathophysiology

Selective deletion of p115-Rho GEF-RhoA in megakaryocyte induces macrothrombocytopenia in mice as well as reduced platelet activation following $G_{\alpha q}$ stimulation, complete inhibition of shape change following $G_{\alpha 12/13}$ stimulation, and defect in granule secretion. RhoA is also important for outside-in mediated signaling as judged by reduced clot retraction. This broad participation to platelet function translates into significant prolongation of the bleeding time and significant protection from thrombosis (Pleines et al. 2012).

Pharmacological Modulation

Development of a RhoA inhibitor has certainly been affected by the bleeding diathesis and macrothrombocytopenia associated with RhoA deletion.

TRPC6

Genetic Modulation and Pathophysiology

Transient receptor potential nonselective calcium channels TRPC6 is an important effector leading to cytosolic Ca^{++}-mediated PS exposure in platelets (Mahaut-Smith 2013) and platelet aggregation (Vemana et al. 2015). Mice deficient in TRPC6 have prolonged tail bleeding time and are protected from arterial thrombosis (Paez Espinosa et al. 2012). This phenotype is believed to stem from a defect in $G_{\alpha q}$ signaling pathway, following thrombin stimulation of platelets. TRPC6 belongs to a class of human transient receptor potential canonical proteins which also contains TRPC1, TRPC3, TRPC4, TRPC5, and TRPC7. These proteins are distributed broadly and are involved in many cellular responses.

Pharmacological Modulation

There have been no reports of drugs targeting TRPC6 for thrombosis indication.

PLCβ

Genetic Modulation and Pathophysiology

Phospholipase Cβ promotes the cleavage of PIP2 into IP3 and DAG following engagement of G protein-coupled receptors. Deficiency in $PLC\beta_2$ in mice is accompanied by defective thrombus stability in vivo (Lian et al. 2005). Interestingly, patients presenting approximately a third of $PLC\beta_2$ have been described and show a mild inherited bleeding disorder together with a defective platelet aggregation process (Lee et al. 1996).

Pharmacological Modulation

Since intermediate levels of $PLC\beta_2$ are associated with bleeding risks, this target may not be suited for development of a safe antithrombotic.

sGC

Genetic Modulation and Pathophysiology

Soluble guanylyl cyclase (sGC) and cGMP-dependent protein kinase (PKG) have been reported to have biphasic effects in platelets, characterized by an initial stimulatory boost followed by inhibitory activities (Li et al. 2003; Zhang et al. 2011). Platelet-specific sGC-deficient mice have prolonged bleeding time (Zhang et al. 2011). PKG inhibitors affect GPIbα and thrombin/PARs signaling pathways leading to $\alpha_{IIb}\beta_3$ activation (Li et al. 2006). Due to its dual activity, development and use of a sGC/PKG inhibitor would have to be carefully evaluated in clinic in order to determine its relative efficacy and safety profiles.

Pharmacological Modulation

Vericiguat has been studied in chronic heart failure patients. Riociguat, a sGC stimulator, is currently being studied in patients with chronic thromboembolic pulmonary hypertension. It has been approved to treat hypertension and chronic thromboembolic pulmonary hypertension and associated with minor or more severe bleeding episodes which could be attributed to its vascular and platelet effects.

JNK1

Genetic Modulation and Pathophysiology

C-Jun aminoterminal kinase (JNK1) belongs to the serine/threonine MAP kinase family, is found in platelets, and is ubiquitously expressed in proliferating cells (Kumar et al. 2015). JNK1 is activated by thrombin, collagen, and vWF (see Adam et al. 2010). JNK1-deficient mice are characterized by a reduction in thrombotic response triggered by fibrillar collagen, unstable thrombi in vivo, decreased secretion, and prolonged bleeding time (with a tendency to rebleed) (Adam et al. 2010). $JNK1^{-/-}$ platelets have a small defect in PKC activation and TxA_2 synthesis (another JNK isoform is believed to contribute to TxA_2 formation). It has been reported that JNK1 and ERK2 possess complementary roles in collagen-induced platelet

signaling, with ERK2 having both JNK1 dependent and independent activities.

Pharmacological Modulation

There have been no reports of drugs targeting JNK1 for thrombosis indication.

$cPLA_2$

Genetic Modulation and Pathophysiology

Platelets possess secretory and cytosolic phospholipase A2 ($cPLA_2$). $cPLA_2$ releases arachidonic acid (AA) from glycerophospholipids, promoting the release of thromboxane A_2 (TxA_2, a potent platelet agonist) via cyclooxygenase 1 (COX1). Deficiency in $cPLA_2$ is associated with decreased granule secretion and platelet aggregation in response to collagen but also with prolonged bleeding time (Wong et al. 2002). Recently, Yoda and colleagues have shown that another PLA_2 ($iPLA_2\gamma$) plays a role in platelet aggregation in response to ADP and hemostasis (Yoda et al. 2014). A direct impact on primary hemostasis is likely explained by the fact that TxA_2 synthesis (like the release of ADP) is key to the platelet feedback loops enhancing the aggregation reaction (Prevost et al. 2009; Estevez et al. 2013). Deficiency of $cPLA_{2\alpha}$ is also responsible for defective fertility (Bonventre et al. 1997) and may protect from experimental autoimmune encephalomyelitis via inhibition of T-cell differentiation (Marusic et al. 2005).

Pharmacological Modulation

A potent, selective inhibitor of $cPLA_2$ has been developed (giripladib) and showed ability to inhibit platelet adhesion and aggregation on fibrillar collagen (Duvernay et al. 2015). Interestingly, under conditions of low collagen-induced activation (that does not involve $cPLA_2$), it is believed that PLC generates diacylglycerol (DG) which is subsequently the subject of the action of diglyceride lipase (DGL), leading to the release of arachidonic acid. A DGL inhibitor has recently been shown to affect platelet aggregation induced by collagen (Jackson et al. 2013).

PAR Intracellular Domain

Pharmacological Modulation

As an alternative to classical orthosteric inhibitor like vorapaxar, inhibition of PAR1 can be achieved via interference with its signaling pathway (O'Callaghan et al. 2012). Recently, Aisiku and colleagues described a class of antiplatelet agents selectively targeting one signaling pathway downstream of PAR1. The agents called parmodulins selectively interfere with $G_{\alpha q}$ signaling, while sparing the believed cytoprotective effect associated with $G_{\alpha 12/13}$ (Aisiku et al. 2015). Thus, it is possible that disease-specific agents could be developed based on the biased signaling properties of PARs.

Effectors Involved Beyond Thrombosis

Syk

Genetic Modulation and Pathophysiology

Syk is a signaling protein with kinase and scaffolding activities. In mice, Syk deficiency is accompanied by embryonic lethality, anemia, petechial hemorrhages throughout the gut, and impairment of B-cell development (Cheng et al. 1995; Turner et al. 1995). Like SLP-76 (see below), it plays a key role in lymphovenous homeostasis where it maintains separation between blood vessels and lymphatic vessels. In platelets, phosphorylation of Syk has been reported downstream of GPVI, GPIbα, and $\alpha_{IIb}\beta_3$ (Law et al. 1999a, b; Watson et al. 2005), following exposure to high shear stress (Speich et al. 2008), after engagement of FcγRIIA, FcRγ, FcαRI, and the C-type lectin receptor-2 (CLEC-2) (Yanaga et al. 1995; Chacko et al. 1996; Suzuki-Inoue et al. 2006). Syk also promotes the recruitment of leukocytes via regulation of leukocyte rolling and adhesion (Mocsai et al. 2002; Frommhold et al. 2007; Zarbock et al. 2007). Interestingly, $Syk^{+/-}$ mice are protected from thrombosis, and pharmacologic modulation of Syk inhibits thrombosis, restenosis, and atherosclerosis while maintaining normal homeostasis (Andre et al. 2011).

Pharmacological Modulation

Syk inhibitors are being developed for the treatment of rheumatoid arthritis (fostamatinib, Rigel), allergic asthma, oncology (fostamatinib, PRT2070), and immune thrombocytopenia (fostamatinib). R406 is notably being developed for the treatment of refractory ITP based on strong preclinical evidence (Podolanczuk et al. 2009; Reilly et al. 2011). Data also showed that modulation of Syk with a selective inhibitor could safely inhibit thrombosis and the progression of atherosclerosis and restenosis in rodents (Andre et al. 2011; Hilgendorf et al. 2011).

SLP-76

Genetic Modulation and Pathophysiology

The adapter protein Src homology 2 domain-containing leukocyte protein 76 (SLP-76) was originally found in T-cells (Jackman et al. 1995). Mice deficient in SLP-76 exhibit a complete block in T-cell development through a defect in pre-TCR signaling and subcutaneous hemorrhages

(Clements et al. 1999). Maintenance of vascular integrity during inflammation is under the critical influence of platelet ITAM signaling as highlighted by the work of Bergmeier and colleagues (Boulaftali et al. 2013). This is to be related to the original observation made in Syk and SLP-76$^{-/-}$ mice in which separation of blood vessels and lymphatic vessels is impaired (Bertozzi et al. 2010; Finney et al. 2012). Despite the presence of hemorrhage, bleeding time is normal in animals with loss of function of SLP-76 (Clements et al. 1999). Furthermore, Bezman and colleagues showed the existence of key tyrosine residues in SLP-76 for the ITAM and integrin receptor signaling leading to platelet function (Bezman et al. 2008).

Pharmacological Modulation

There are no SLP-76 inhibitors undergoing late-stage development for thrombosis indication.

PLCγ2

Genetic Modulation and Pathophysiology

PLCγ2 is mostly expressed in hematopoietic cells (B-cells, platelets, mast cells, and monocytes/macrophages) where it functions as a key signaling player of immunoglobulin superfamily receptors. PLCγ2 knockout mice have normal blood cell counts but impaired BCR-mediated signaling leading to a *xid*-like immunodeficiency phenotype, decreased platelet reactivity, and some levels of subcutaneous, intraperitoneal, and gastrointestinal hemorrhage (Wang et al. 2000). Remarkably, PLCγ2$^{-/-}$ mice have a prolonged bleeding time, but not the PLCγ2$^{+/-}$ animals (Mangin et al. 2003). Importantly, others have shown that knockout animals do not have prolonged bleeding times in spite of a significant protection from thrombosis (Kalia et al. 2008). Platelets deficient in PLCγ2 have a defect in granule release and aggregation following collagen stimulation [in a concentration-dependent manner (Cho et al. 2003)] but do have a normal response to ADP and thrombin.

Pharmacological Modulation

There are no PLCγ inhibitors undergoing late-stage development for thrombosis indication.

CalDAG-GEFI

Genetic Modulation and Pathophysiology

The Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor (CalDAG-GEFI) plays a key role in platelet activation. It activates the small GTPase Rap1, enhancing talin binding to $\alpha_{IIb}\beta_3$ and allowing full activation of the fibrinogen receptor. Mutations in RASGRP2 prevent Rap1 activation, leading to a severe platelet aggregation defect and bleeding diathesis (see Canault et al. 2014; Nurden and Nurden 2015). CalDAG-GEFI deficiency has been shown to protect from ITAM-dependent thrombotic thrombocytopenia (Stolla et al. 2011; Amirkhosravi et al. 2014) and thrombosis (Bergmeier et al. 2007). Defective expression of CalDAG-GEFI was also suggested as the cause of leukocyte adhesion deficiency (LAD-III syndrome), a clinical disorder characterized by impaired activation of integrins in platelets and leukocytes (Bergmeier et al. 2007; Pasvolsky et al. 2007). However, all cases of LAD-III are caused by mutations in the gene Fermt3. CalDAG-GEFI also plays a role in leukocyte rolling and chemotaxis (Carbo et al. 2010; Stadtmann et al. 2011), possibly in the pathology of Huntington's disease (Crittenden et al. 2010) and motor complications (Crittenden et al. 2009).

Pharmacological Modulation

A safe selective inhibitor of CalDAG-GEFI will require that effective target engagement levels (thrombosis) will be different from those leading to bleeding (and other biology). There are no known inhibitors of CalDAG-GEFI in development.

Rap1b

Genetic Modulation and Pathophysiology

Rap1b-deficient mice have 85 % incidence of embryonic and perinatal lethality (with ~40 % having abdominal, cranial, and hepatic bleeding). Generation of Rap1b-null bone marrow chimeras revealed that platelet Rap1b is involved in primary hemostasis, a phenomenon likely to be attributed to its broad role in platelet activation. Indeed, Rap1b$^{-/-}$ platelets exhibit and impaired aggregation response to all platelet agonists (thrombin, collagen, and ADP (including calcium ionophore), independently of the agonist concentration (Chrzanowska-Wodnicka et al. 2005). In addition, Rap1b is also mediating $\alpha_{IIb}\beta_3$ outside-in signaling, explaining the strong protection from thrombosis (Chrzanowska-Wodnicka et al. 2005). Besides a critical role in platelet function, Rap1b has been shown to play major roles in neutrophil chemotaxis (Kumar et al. 2014), B- and T-cell functions (Chu et al. 2008), vascular tone, and blood pressure (Lakshmikanthan et al. 2014).

Pharmacological Modulation

There are no known inhibitors of Rap1b in development for the treatment or prevention of thrombosis.

Pyk2

Genetic Modulation and Pathophysiology

In platelet signaling, Pyk2 (also named the Ca^{++}-dependent tyrosine kinase) promotes Lyn and Fyn activation following thrombin stimulation and acts as a positive feedback loop with/on the Src family kinase (Canobbio et al. 2015). It is also activated downstream of GPIbα and integrins, and its deficiency is associated with prolonged bleeding and protection from thrombosis (Canobbio et al. 2013). Pyk2 biology extends beyond platelet function as it plays an important role in osteoblast and bone formation (Cheng et al. 2013). Pyk2 is also being considered as a target for diverse cancers, including multiple myeloma (Meads et al. 2015).

Pharmacological Modulation

There are no known inhibitors of Pyk2 in development for the treatment or prevention of thrombosis.

Disabled-2

Genetic Modulation and Pathophysiology

Platelet-specific deletion of disabled-2 in mice has revealed a prolonged bleeding time and inhibition of thrombus formation due to a diminished response to thrombin stimulation (Tsai et al. 2014). The inhibition affects $G_{\alpha 12/13}$-mediated RhoA activation and Akt and mTOR phosphorylation and does not seem to interfere with $G_{\alpha q}$ signaling. Pyk2 plays a role in endocytosis, cell surface receptor turnover, and signaling, and its downregulation has been associated with (notably) fibrotic processes, epithelial-to-mesenchymal transition (Martin et al. 2010a, b), and tumorigenesis (Yang et al. 2006).

Pharmacological Modulation

While a disabled-2-derived peptide has been used in perfusion chamber studies in which it demonstrated anti-aggregatory activities, modulation of disabled-2 in vivo has not been tested in the context of thrombosis (Xiao et al. 2012). There are no known inhibitors of Pyk2 in development for the treatment or prevention of thrombosis.

Targets from the Outside-In Signaling Pathway and Their Pharmacological Modulation

The first indication that targeting the $\alpha_{IIb}\beta_3$ outside-in signaling pathway may have an acceptable therapeutic window came from the observation of Law and colleagues who showed that a cytoplasmic tyrosine motif was associated with protection from thrombosis with minor effect on bleeding (Law et al. 1999a, b). Several key effectors have been identified.

$G_{\alpha 13}$

A myristoylated ExE motif peptide inhibiting the $G_{\alpha 13}$-integrin interaction demonstrated the ability to inhibit spreading on the integrin ligand and to inhibit the second wave of platelet aggregation. More importantly, it blocked thrombosis in vivo without prolonging the bleeding time (Shen et al. 2013).

SFK

A peptide derived from the c-Src binding sequence of β_3 has shown selectivity for the outside-in pathway and provided protection from thrombosis although this was accompanied by an increase in bleeding (Su et al. 2008; Ablooglu et al. 2009).

Kindlin-2

Kindlin-2 is a cytoskeletal protein that has been implicated in β_3 integrin signaling pathway (Ma et al. 2008; Liao et al. 2015) and which deficiency is embryonically lethal in mice. Heterozygous mice are protected from thrombosis but also present defect in hemostasis, a phenomenon that has been tentatively related to dysregulation of ADP/AMP catalytic enzymes at the surface of endothelial cells (Pluskota et al. 2013). There are no known inhibitors of Kindlin-2 in development for thrombosis indications.

Lnk

Lnk (lymphocyte adaptor protein) inhibits thrombopoietin-mediated signaling, leading notably to increased platelet production in Lnk-deficient animals. Null mice have a tendency to rebleed and are protected from thrombosis despite presenting a significant increase in platelet count (Takizawa et al. 2010). Lnk main function in thrombosis seems to be in the outside-in signaling pathway where it mediates reorganization of the cytoskeleton. There are no known inhibitors of Lnk in development for thrombosis indications.

PDK1

PDK1 (phosphoinositide-dependent protein kinase 1) is activated by PI3K (via binding to PIP3) and induces

phosphorylation and activation of Akt. Using a platelet-specific PDK1 deletion strategy, PDK1 was demonstrated to enhance thrombin-induced platelet aggregation and outside-in signaling (Chen et al. 2013). PDK1 functions go beyond thrombosis as it participates in multiple cellular functions (cell growth/proliferation/survival), β-adrenergic response and overall cardiac homeostasis (Ito et al. 2009). There are no known inhibitors of PDK1 in development for thrombosis indications.

ILK

Like PDK1, ILK (integrin-linked kinase) regulates multiple cellular functions and associates with β3 (as well as β1) integrin. Mice deficient in ILK have a defect in thrombus formation and α-granule release and exhibited enhanced bleeding time (Tucker et al. 2008). It is also an important contributor to cardiomyocyte homeostasis (like PDK1) and renal homeostasis.

Take Home Message

Comments

1. Some effectors of platelet signaling exist that are involved in thrombosis but not in primary hemostasis.
2. Most effectors possess functions beyond a single signaling pathway inside the platelet or in a different cellular compartment.
3. Some effectors have ubiquitous role in platelet function (i.e., CalDAG-GEFI and Rap1b) and have a major impact on primary hemostasis; intermediate levels of inhibition may provide protection from thrombosis at no cost on bleeding.
4. Some effectors have an impact beyond platelet function. In this category, we can group Syk, SLP-76, and PLCγ2 as their deficiencies are associated with defective platelet function and also vascular defects, but again, pharmacologic modulation at intermediate levels could provide a viable therapeutic window.
5. The severity of the platelet defect (on secretion and $\alpha_{IIb}\beta_3$ activation) is often, but not always, associated with bleeding intensity. For example, deficiency in or modulation of RhoG, PI3Kp85, Btk, PAK, and PKCα severely impairs platelet function at limited or no cost on bleeding.
6. There is a need for selective tool compounds (across animal species) to better characterize and de-risk targets early.
7. Investigators should document the phenotype associated with heterozygous mice, as it would provide valuable information to the industry (related to the validity of the target and efficacious target engagement levels).
8. We encourage investigators to study the signaling pathways in presence of concomitant antithrombotic therapies (antiplatelet and/or anti-coagulants) in order to understand the physiology associated with dual or triple inhibition.

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Part VII

Therapy

Antiplatelet Therapy in Patients with Coronary Artery Disease

Harsh Patel and Neal S. Kleiman

Abstract

Treatment with aspirin has been a cornerstone of acute therapy and of secondary prevention for patients with active coronary artery disease, and in particular those with acute myocardial infarction, acute coronary syndromes, and those undergoing either percutaneous or surgical revascularization. Studies within the past decade have highlighted the added benefit of treatment with antagonists of $P2Y_{12}$ among those with acute coronary syndromes and those undergoing percutaneous revascularization. The optimal duration of treatment appears to be at least 15 months after the onset of the acute coronary syndrome or 30 months for patients who undergo intracoronary implantation of a drug eluting stent, however studies with newer generation stents in selected patients have indicated that shorter durations may well be acceptable. For patients who also have a need for an oral anticoagulant, particularly those with atrial fibrillation or with mechanical prosthetic heart valves, the anti-platelet drug regimen is much less clear. Clinical trials of new regimens in this situation continue to evolve and have indicated that prolonged duration of a triple drug regimen (oral anticoagulant, aspirin, and a $P2Y_{12}$ antagonist) is associated with an increased risk of hemorrhage, and that a regimen that excludes aspirin may be acceptable.

Introduction

Antiplatelet drugs have become a cornerstone of therapy in cardiology. The value of aspirin in reducing thrombotic events in patients with atherosclerotic vascular disease has been noted since the 1950s. Several trials over the past few decades have firmly established its role in both primary and secondary prevention of cardiovascular events resulting from atherosclerotic vascular disease. Several oral $P2Y_{12}$ antagonists have also been proven in randomized studies to provide incremental benefit in addition to aspirin in secondary prevention of thrombotic events in high-risk subgroups. However, the optimal duration of dual antiplatelet therapy is still controversial and remains an area of investigation. Ultimately the optimal duration is likely to be dependent on several patient-specific factors that determine risk of thrombotic events, as well as bleeding. Finally, the role of antiplatelet drugs in non-atherosclerotic disorders such as atrial fibrillation is limited, as most studies have not shown benefit in reducing thrombotic events in comparison to anticoagulants or even placebo.

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Antiplatelet Therapy for Secondary Prevention in Stable Coronary Artery Disease

Aspirin

Aspirin is an important cornerstone of therapy in reducing cardiovascular morbidity and mortality in patients with known or established stable coronary artery disease. The mechanisms of aspirin's antiplatelet action are reviewed in Patrono (2017). Several studies have firmly established the role of aspirin in reducing recurrent myocardial infarction, stroke, and vascular death in patients with known symptomatic coronary artery disease. In a meta-analysis by the

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Antithrombotic Trialists' Collaboration of 195 randomized trials including more than 135,000 patients with prior evidence of cardiovascular disease, long-term antiplatelet therapy (primarily with aspirin) in comparison to placebo was found to reduce the combined outcome of nonfatal myocardial infarction, nonfatal stroke, or vascular death by 33 % in patients with stable coronary artery disease and by 53 % in patients who underwent percutaneous coronary intervention (Antithrombotic Trialists' Collaboration 2002). The investigators reported no significant difference in safety or efficacy between doses of 75–150 mg and 160–325 mg. The case for primary prevention is more complex and is still evolving; current guidelines emanating from professional societies provide conflicting recommendations. An interesting potential paradox was reported by the investigators of the Women's Health Study who noted a 24 % relative risk reduction in the frequency of ischemic stroke but not myocardial infarction among women assigned to low-dose aspirin (Ridker et al. 2005), in contradistinction to a reduction in myocardial infarction but not stroke among men enrolled in prior trials of aspirin as secondary prevention, although it is questionable whether this observation can be validated in studies involving large numbers of patients. Given the pathophysiology of atherosclerotic disease (as it is currently understood) and the mechanism of aspirin's action, these reports remain rather vexing. However, the overall clinical consideration in using aspirin for the primary prevention of atherosclerotic-mediated events requires that one understand the balance between event prevention and the risk of bleeding, particularly among patients treated with antiplatelet drugs. As the role of aspirin in primary prevention still remains unsettled, it is interesting to note an analysis reported by the European Society of Cardiology Working Group on Thrombosis, which proposed that given the competing risks, ischemic events, and hemorrhage, aspirin must be given to patients whose 10-year risk of death, myocardial infarction, or stroke exceeds 20 % (Halvorsen et al. 2014).

Clopidogrel

Clopidogrel has also been studied extensively for secondary prevention of cardiovascular events in patients with known stable coronary artery disease. In the CAPRIE trial which randomized 19,185 patients with recent myocardial infarction or recent ischemic stroke or symptomatic peripheral arterial disease to treatment with clopidogrel 75 mg or aspirin 325 mg daily, patients treated with clopidogrel were found to have modest and marginally significant reduction in stroke, myocardial infarction, or vascular death at 2 years (5.32 % versus 5.83 %) as well as lower rates of major gastrointestinal bleeding (CAPRIE Steering Committee 1996). Whether the latter toxicity would have been different between the two treatments had a lower aspirin dose been selected is unknown. Similarly, the Antithrombotic Trialists' Collaboration also reported a small reduction in serious cardiovascular events in patients taking clopidogrel compared to aspirin for secondary prevention (Antithrombotic Trialists' Collaboration 2002). These trials established the value of clopidogrel as an alternative to aspirin (such as in patients with intolerance or allergy to aspirin) for secondary prevention of cardiovascular events.

Other studies have evaluated the incremental benefit of adding clopidogrel to aspirin in reducing cardiovascular events. In the Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial, 15,603 patients with clinically evident cardiovascular disease (i.e., symptomatic coronary artery disease, peripheral arterial disease, or cerebrovascular disease) or multiple risk factors were randomized to receive clopidogrel 75 mg versus placebo in addition to low-dose aspirin 75–162 mg daily (Bhatt et al. 2006). The study found no difference in composite of myocardial infarction, stroke, or cardiovascular death between the two groups (6.8 % with combination therapy versus 7.3 % with aspirin alone). There was also no significant difference in rates of major bleeding between the two treatment groups in the overall study population. However, in the subgroup of patients with clinically evident cardiovascular disease (12,153 patients), clopidogrel compared to placebo was associated with significant reduction in the primary end point (6.9 % versus 7.9 %, respectively) and no difference in the rate of major bleeding. By contrast, in the subgroup of patients with multiple cardiovascular risk factors but no overt cardiovascular disease, clopidogrel compared to placebo was associated with no difference in primary end point but with increased rates of severe bleeding (2.0 % versus 1.2 %, respectively). Other subsequent post hoc analyses indicated a reduction in events among high-risk patients with diabetes and those with established atherosclerosis (Bhatt et al. 2002, 2007; Ringleb et al. 2004).

Despite the overall negative results of the CHARISMA trial, other studies have clearly shown the benefit of dual antiplatelet therapy for secondary prevention in patients with stable coronary artery disease, especially after percutaneous coronary intervention. The rationale for dual antiplatelet therapy after coronary stent placement relates to the thrombogenicity of exposed metal struts which sit adjacent to mechanically disrupted atheromata. Soon after the adoption of balloon angioplasty, Steele et al. observed in a pig carotid injury model that platelet deposition at the site of injury began within the first hour after balloon injury (Steele et al. 1985). This report was followed by two small clinical studies indicating that aspirin led to reductions in clinical events after balloon angioplasty (Schwartz et al. 1988; Barnathan et al. 1987). Since then, coronary stenting has virtually replaced balloon angioplasty for percutaneous revascularization in patients with coronary artery disease. Several

studies have clearly indicated that adding a $P2Y_{12}$ antagonist to aspirin is mandatory following coronary stent implantation. The first such observation was made with ticlopidine in combination with aspirin (Colombo et al. 1995). Interestingly, although there was no control group and the observations were made in a single center, these observations had a profound effect on the practice of cardiology and are nearly single-handedly responsible for the clinical acceptance of intracoronary stents, even before this report was published. Since that time, the combination of aspirin with a $P2Y_{12}$ antagonist has been referred to as "dual antiplatelet therapy" (DAPT). Multiple studies early after the development of bare-metal stenting showed that rates of stent thrombosis and myocardial infarction in the weeks and months after implantation were significantly lower in patients treated with combination of aspirin and ticlopidine compared to aspirin alone or in combination with oral anticoagulants (Leon et al. 1998; Urban et al. 1998). Thus dual antiplatelet therapy emerged rapidly as the standard of care after coronary stenting. Because of its tolerability and lack of myelosuppresive complications (profound neutropenia), clopidogrel quickly replaced ticlopidine as the second antiplatelet agent (in addition to aspirin) after it was found to have similar efficacy and superior safety/tolerability profile in the CLASSICS trial (Bertrand et al. 2000). No studies have compared dual antiplatelet therapy with aspirin and clopidogrel directly to aspirin monotherapy after drug-eluting stent placement due to the strong evidence favoring dual antiplatelet therapy in patients with bare-metal stents.

Ticagrelor

The newer oral $P2Y_{12}$ antagonist ticagrelor was originally approved in 2011 for use in combination with aspirin in patients with acute coronary syndromes (ACS) based on the results of the PLATO trial. In patients with ACS, ticagrelor compared with clopidogrel led to a 16 % relative risk reduction in a composite end point consisting of cardiovascular death, myocardial infarction, or stroke (9.8 % versus 11.7 %) as well as a significant reduction in stent thrombosis among patients who had been treated with intracoronary stents (Cannon et al. 2010). In addition, the rate of vascular mortality was also reduced (4.0 % versus 5.1 %) in a hierarchical analysis. While many of these effects are explicable on the basis of more potent and more rapid onset of ticagrelor's antiplatelet effect compared with clopidogrel, a possible extra-platelet effect has been postulated, largely as a result of ticagrelor's inhibition of cellular uptake of adenosine leading to higher local levels of the compound (Armstrong et al. 2014). Ticagrelor given prior to angiography has become increasingly popular in patients with ACS given prior to coronary angiography in part because of its rapid onset of action, but also because its more rapid offset of action is more convenient in patients who may ultimately be found to require coronary bypass surgery (Gurbel et al. 2009). Recently, the benefit of dual antiplatelet therapy with aspirin and reduced-dose (60 mg twice daily) ticagrelor in patients with stable coronary artery disease was evaluated in the PEGASUS-TIMI 54 trial (Bonaca et al. 2015). The study enrolled 21,162 high-risk patients who had had a myocardial infarction more than 1 year previously and randomized them to treatment with ticagrelor 90 mg twice daily, 60 mg twice daily, or placebo. All patients were treated with low-dose aspirin. The majority (83 %) of patients had a history of percutaneous coronary intervention. At an average of 3 years of follow-up, the primary composite end point of cardiovascular death, myocardial infarction, or stroke was lower in the patients receiving ticagrelor at either dose compared to placebo (7.85 %, 7.77 %, and 9.04 %, respectively). The reduction in primary end point was driven mainly by lower rates of myocardial infarction and stroke in patients on ticagrelor compared to placebo with no significant difference in rate of death. TIMI major bleeding was also significantly higher in patients receiving ticagrelor compared to placebo (2.6 %, 2.3 %, and 1.06 %, respectively), but there were no differences in rates of fatal bleeding or intracranial hemorrhage.

Antiplatelet Therapy in Patients with Unstable Angina

Plaque disruption followed by platelet deposition and thrombosis is considered to be the pathophysiologic hallmark of the acute coronary syndromes ST segment elevation myocardial infarction, non-ST segment elevation myocardial infarction, and unstable angina. The degree of vascular obstruction can vary from complete arterial obstruction, which results in confluent areas of myocardial necrosis, as seen in ST elevation myocardial infarction; incomplete obstruction, resulting in non-ST elevation myocardial infarction or unstable angina; or embolization of platelet fibrin aggregates resulting in patchy myocardial necrosis. Numerous professional societies have published guidelines for the administration of antiplatelet drugs to patients with acute coronary syndromes (ACS). Those most often cited are products of the European Society of Cardiology and of the American College of Cardiology/American Heart Association. Since the guidelines undergo revision quite frequently as new trial data are published, the reader is referred to either of these two dynamic sources for current specific recommendations on drug dosing in various patient groups. The following section will provide a brief overview on the topic. Considerable attention has been devoted to interdicting the platelet-vascular interaction, for the past five decades. The Veterans Affairs Cooperative Study first studied aspirin 324 mg in a double-blind placebo-controlled trial in 1266 men with unstable angina, whose last

episode of pain had occurred within the prior week, and observed a 51 % reduction in the relative risk of myocardial infarction at 12 weeks, accompanied by a 51 % reduction in mortality (Lewis et al. 1983). Strikingly similar results were observed by Theroux et al. in a study of 479 patients treated at a mean of 7.9 h after the last episode of pain (Theroux et al. 1988). These two studies, along with the ISIS-2 study demonstrating a 23 % reduction in mortality at 5 weeks in patients with ST segment elevation myocardial infarction among those randomized to receive aspirin rather than standard therapy (ISIS-2 1988), firmly established the role of aspirin as first-line therapy among patients with acute coronary syndromes. Additional supporting evidence was provided by the study by Theroux, in which heparin was superior to aspirin, but aspirin was able to prevent the syndrome of disease reactivation that occurred when heparin was withdrawn (Theroux et al. 1992). As $P2Y_{12}$ antagonists were developed, a small trial was performed in which ticlopidine 250 mg twice daily was compared with standard treatment (aspirin was not given) in 652 patients with transient ST segment depression or T-wave inversion within 48 h of admission to the coronary care unit. At 24 weeks, a 46.8 % reduction was observed in the composite of vascular death or fatal myocardial infarction, accompanied by a 46.1 % reduction in nonfatal myocardial infarction (Balsano et al. 1990). However, because of gastrointestinal side effects and the risk of neutropenia, ticlopidine was not commonly used as a standard therapy in unstable angina. Ten years later, the Clopidogrel in Unstable Angina to Prevent Recurrent Events (CURE) trial enrolled 12,562 patients within 24 h of a non-ST elevation acute coronary syndrome and randomized them to receive either daily clopidogrel 75 mg and aspirin or aspirin alone for 15 months. Treatment with clopidogrel led to a 20 % relative reduction in cardiovascular death, myocardial infarction, or stroke (11.4 % versus 9.3 %) (Yusuf et al. 2001). Subsequent trials with the newer $P2Y_{12}$ antagonists prasugrel (Wiviott et al. 2007) and ticagrelor (Wallentin et al. 2009) have demonstrated that they are superior to clopidogrel at reducing ischemic events in patients with acute coronary syndromes when started soon after hospitalization and continued (for 9 months in the case of ticagrelor or 15 months in that of prasugrel). The increased risk of bleeding when it is given prior to coronary angiography (Montalescot et al. 2013) has limited the indication for prasugrel to patients undergoing emergency angiography for ST segment elevation myocardial infarction and those undergoing percutaneous coronary interventions, while the indication for ticagrelor does not require knowledge of coronary anatomy. While theoretically appealing, prehospital use of ticagrelor in patients with ST segment elevation myocardial infarction was not shown to be superior to in-hospital administration of the drug (Montalescot et al. 2014). It is important to recognize that morphine which is frequently used to provide analgesia to patients with myocardial infarction inhibits gastrointestinal motility and thus may inhibit absorption of antiplatelet medications and thus impair their activity (Parodi et al. 2015). Accordingly, the intravenous adenosine triphosphate (ATP) analog cangrelor was developed to provide rapid $P2Y_{12}$ inhibition, particularly in patients with acute coronary syndromes in whom oral administration is either not feasible or unlikely to be effective. The onset of action of cangrelor occurs within minutes of initiating an intravenous dose and subsides within an hour after terminating it. Cangrelor was studied in the CHAMPION trials in patients with both stable and unstable manifestations of coronary artery disease. Cangrelor was able to reduce periprocedural myocardial infarction in patients undergoing intracoronary stent implants. Although there was no formal statistical interaction between cangrelor treatment and presentation with stable angina versus acute coronary syndromes (Bhatt et al. 2013), intuitively, one would suspect that the utility of the drug is greatest in the latter setting because of limitations in the feasibility of oral drug loading. Caution should be taken when transitioning patients from cangrelor to oral clopidogrel because of competition for the $P2Y_{12}$ binding site between cangrelor and the active metabolite of clopidogrel (Steinhubl et al. 2008). Consequently, the clopidogrel loading dose should be administered at the end of the cangrelor infusion. Similar observations have been made in transitioning patients between cangrelor and prasugrel, but less so in transitioning patients to ticagrelor (Center for Drug Evaluation and Research 2015).

Antagonists of integrin $\alpha_{IIb}\beta_3$ [often referred to as glycoprotein (GP) IIb/IIIa inhibitors] are also used frequently in patients with acute coronary syndromes. The initial studies with abciximab were performed in patients undergoing percutaneous coronary interventions and showed reductions in myocardial infarction, particularly among patients with acute coronary syndromes (ACS) (The EPIC Investigators 1994; The EPISTENT Study Group 1998). Similar findings were reported with the peptide eptifibatide (The IMPACT II Investigators 1997) and the peptidomimetic tirofiban (The RESTORE Investigators 1997). These drugs were used in combination with heparin. An important early finding was that it was necessary to use a reduced dose of heparin (on the order of 60–70 mg/kg rather than the 100 mg/kg dose originally used) in order to minimize the bleeding excess produced by $\alpha_{IIb}\beta_3$ antagonists (The EPILOG Investigators 1997). Extension of these findings to patients treated medically was achieved in broad-based trials of eptifibatide and tirofiban, in which the medication was begun soon after diagnosis (commonly referred to as "upstream" therapy), regardless of whether coronary intervention was planned (The PRISM PLUS Study Group 1998; The PURSUIT Trial Investigators 1998). A similar trial with abciximab did not show a clinical benefit (Simoons 2001). The effect of these treatments when given upstream is often regarded as greatest in patients who subsequently underwent percutaneous coronary interventions (Kleiman et al. 2000), although it

should be noted that such analyses generally represent post-randomization (or "improper") subgroups and should be regarded as statistically unreliable. Based on such observations, a number of oral $\alpha_{IIb}\beta_3$ antagonists were developed but were shown in trials of extended duration of therapy to be associated with both increased bleeding risk and a paradoxical increase in death and myocardial infarction which was believed to be a consequence of platelet activation through outside-in signaling (Chew et al. 2001). The use of $\alpha_{IIb}\beta_3$ antagonists has decreased in recent years, partly based on shortened periods of hospitalization preceding coronary angiography and revascularization, as well as comparisons with the direct thrombin bivalirudin showing less frequent bleeding without an increment in ischemic events when bivalirudin was substituted for the combination of heparin and an $\alpha_{IIb}\beta_3$ antagonist (Stone et al. 2006).

Duration of Dual Antiplatelet Therapy

While it is extremely clear that dual antiplatelet therapy after coronary stenting reduces rates of acute (<24 h) and subacute (<30 days) stent thrombosis and myocardial infarction, the optimal duration of therapy remains unclear. Initial observations after development of coronary stenting consistently showed elevated risk of stent thrombosis for 1 year after stent placement, leading to the general practice of 1 year of dual antiplatelet therapy (Levine et al. 2011). However, in patients with drug-eluting stents, evidence supports the persistence of risk of stent thrombosis even beyond the first year (Mauri et al. 2007). Some observational studies have suggested a possible reduction in rates of myocardial infarction with extended-duration dual antiplatelet therapy after drug-eluting stents (Eisenstein et al. 2007). This was particularly the case in patients who had been treated with first-generation sirolimus-eluting and paclitaxel-eluting stents. Current-generation drug-eluting stents have thinner strut frames and antiproliferative drugs that are embedded in polymers that are less likely to result in vascular inflammation. Several studies indicate that stent thrombosis rates are significantly lower with current-generation drug-eluting stents (Camenzind et al. 2014; Sarno et al. 2014). However, several small open-label randomized trials have shown no significant reduction in rates of myocardial infarction but increased risk of harm due to bleeding with longer therapy (Collet et al. 2014; Park et al. 2010; Valgimigli et al. 2012).

Recently, a large randomized double-blinded trial, the DAPT study, reported continued benefit of dual antiplatelet therapy (with aspirin and either clopidogrel or prasugrel) for up to 30 months after drug-eluting stent in patients who had not had major bleeding in the first 12 months of therapy (Mauri et al. 2014). The study randomized 9961 patients who had undergone percutaneous coronary intervention with drug-eluting stent (38 % for stable coronary disease) 12 months earlier to either continued treatment with aspirin plus thienopyridine or aspirin plus placebo for another 18 months. Notably, patients who needed repeat revascularization or developed recurrent myocardial infarction or bleeding in the first year after index stent placement were excluded. At end of follow-up, the study reported significantly lower rates of stent thrombosis (0.4 % versus 1.4 %) and the composite of death, myocardial infarction, and stroke (4.3 % versus 5.9 %) but higher rates of moderate or severe bleeding (2.5 % versus 1.6 %) in patients receiving extended dual antiplatelet therapy. While cardiovascular mortality was similar in both groups, overall mortality was slightly higher in patients receiving dual antiplatelet therapy (2 % versus 1.5 %) due to higher rates of noncardiac deaths. Prespecified subgroup analysis showed that the benefit of longer-duration dual antiplatelet therapy seemed to be greatest among patients who received first-generation (sirolimus or paclitaxel eluting) stents. Among those who received second-generation (everolimus or zotarolimus eluting) stents, the relative reductions were similar, although the absolute event rates (and therefore absolute reductions) were considerably lower. Notably, there was also evidence of greater benefit of therapy (lower rates of stent thrombosis and major adverse cardiovascular and cerebrovascular events) in patients who underwent stenting for acute coronary syndrome compared to stable coronary artery disease (Yeh et al. 2015). This finding of increased benefit of extended-duration dual antiplatelet therapy in high-risk patients was also corroborated in the recent PEGASUS trial which only enrolled patients with history of myocardial infarction.

Conversely, several smaller trials have also investigated the value of shorter duration (<12 months) of dual antiplatelet therapy after second-generation drug-eluting stents in patients with low-risk features (Feres et al. 2013; Gwon et al. 2012; Kim et al. 2012; Colombo et al. 2014; Gilard et al. 2015; Schulz-Schüpke et al. 2015). All of these abbreviated-term trials had key limitations including low event rates, slow enrollment, and premature termination, tempering their generalizability. A meta-analysis including several of these trials reported no significant difference in rate of major adverse cardiovascular events or mortality but higher rates of bleeding with standard duration (12 months) compared to short duration (3–6 months) of dual antiplatelet therapy (Palmerini et al. 2015a).

Other larger meta-analyses of up to ten trials which evaluated varying durations of dual antiplatelet therapy (from 3 months to 36 months) have been reported, generally with similar conclusions (Palmerini et al. 2015b; Spencer et al. 2015; Giustino et al. 2015). For example, one such meta-analysis including 32,135 patients showed that patients treated with longer-duration dual antiplatelet therapy had lower rates of stent thrombosis and myocardial infarction, though the magnitude of this effect was attenuated in patients

with second-generation drug-eluting stents (Giustino et al. 2015). Risk of clinically significant bleeding was significantly higher in patients taking longer-duration dual antiplatelet therapy. Notably, there was a numerically higher rate of all-cause mortality, which did not reach statistical significance, in patients on long-term dual antiplatelet therapy. The meta-analysis had important limitations including lack of patient-level data, which precludes adjustment for the variable duration and discontinuation regimens of dual antiplatelet therapy in the included trials. Also, risk/benefit analysis of longer-duration dual antiplatelet therapy with regard to the increased risk of bleeding versus decreased risk of stent thrombosis cannot be performed due to lack of information regarding clinical severity of such events. Finally, the significant heterogeneity of the patients in the various trials in terms of baseline risk profile limits generalizability to patients at elevated risk of cardiovascular events.

Udell et al. recently reported a meta-analysis of long-term dual antiplatelet therapy for secondary prevention in patients with prior history of myocardial infarction (Udell et al. 2016). The meta-analysis included 33,435 patients from one trial of patients with prior myocardial infarction (PEGASUS) and five trials with subgroups of patients with prior myocardial infarction (PRODIGY, DAPT, DES-LATE, CHARISMA, and ARCTIC-Interruption). After an average of 31 months of follow-up, patients randomized to longer-duration dual antiplatelet therapy had lower rates of major adverse cardiovascular events including cardiovascular death, myocardial infarction, stroke, and stent thrombosis compared to aspirin alone. There was an increased risk of major bleeding but not fatal bleeding and no difference in all-cause mortality with longer-duration dual antiplatelet therapy.

Thus, the optimal duration of dual antiplatelet therapy for secondary prevention after drug-eluting stents remains controversial. As described above, several trials and meta-analyses have shown a decreased risk of ischemic events but increased risk of bleeding and no clear difference in all-cause mortality with prolongation of dual antiplatelet therapy. Consequently, the decision to continue dual antiplatelet therapy after the first year should probably be individualized and incorporate analysis of patient-specific risk of bleeding as well as risk of ischemic events.

Antiplatelet Therapy for Primary Prevention in Patients with Stable Coronary Artery Disease

Aspirin

Although aspirin has a well-defined role in patients with established cardiovascular disease, its role in primary prevention of coronary events in patients with multiple risk factors is uncertain. A 2009 meta-analysis of six large trials, including more than 95,000 patients without established cardiovascular disease but at elevated risk, showed that aspirin at doses between 75 and 500 mg per day was associated with a significant reduction in rate of first myocardial infarction (0.18 versus 0.23 % per year) but also significant elevation in rate of major bleeding (0.10 versus 0.07 % per year) with no significant difference in stroke or cardiovascular mortality (Antithrombotic Trialists' Collaboration 2009). A more recent analysis, which added patients from three additional trials, also reported similar conclusions (Seshasai et al. 2012). Notably, nearly 80 % of the patients in these meta-analyses came from the Physicians' Health Study, the Women's Health Study, and the Hypertension Optimal Treatment trial, which included patients with low-moderate risk based on an average 10-year risk of a first event of <5 % (Antithrombotic Trialists' Collaboration 2009). Due to the low baseline event rates in these trials, it may not be surprising that in the overall population, the net clinical benefit of aspirin did not outweigh the associated increase in bleeding risk. However, in the small subset of patients with high baseline risk, the net cardiovascular benefit of aspirin did appear to outweigh the bleeding risk (Seshasai et al. 2012).

Thus, given the current evidence, the broad use of aspirin for primary prevention in all patients with cardiovascular risk factors cannot be supported. However, aspirin may be beneficial in certain patients at particularly elevated risk of ischemic events without a high risk of bleeding. Several ongoing randomized trials including ARRIVE and ASPREE which have enrolled patients with moderate to high risk of first cardiovascular event (average 10-year risk of 10–19 %) may help identify subgroups of patients that may benefit from aspirin for primary prevention.

Antiplatelet Therapy for Prevention of Thromboembolism in Patients with Atrial Fibrillation

Aspirin Versus Warfarin

Systemic thromboembolism arising from the left atrium is a major cause of morbidity and mortality in patients with any form of atrial fibrillation. Even though today anticoagulants are considered the standard of care for reducing the risk of thromboembolic complications in patients with atrial fibrillation, the role of antiplatelet agents has also been evaluated in several trials. In the late 1980s–early 1990s, several placebo-controlled trials were conducted to address the clinical equipoise regarding the absolute and comparative effectiveness of warfarin and aspirin in reducing thromboembolic complications in patients with atrial fibrillation. The Stroke Prevention in Atrial Fibrillation (SPAF-I) study randomized 1330 patients with non-valvular atrial fibrillation to either warfarin dose adjusted to INR 2.0–4.5, aspirin 325 mg daily,

or placebo if they were eligible for anticoagulation or if ineligible for anticoagulation to aspirin 325 mg daily or placebo (Stroke Prevention in Atrial Fibrillation Investigators 1991). After mean follow-up of 1.3 years, the study reported that both warfarin and aspirin significantly reduced rate of primary end point (stroke or systemic embolism) compared to placebo (2.3 %, 3.6 %, and 7.4 % respectively). Despite the nominally larger reduction in primary end point with warfarin compared to aspirin, the study was not adequately powered to detect a significant difference between the two therapies. A follow-up study, SPAF-II, compared warfarin dose adjusted to INR 2.0–4.5 with aspirin 325 mg daily in two parallel trials involving 715 patients aged 75 years or less and 385 patients aged >75 years (Stroke Prevention in Atrial Fibrillation Investigators 1994). After a mean follow-up of 2.0 years, warfarin was significantly more effective than aspirin in reducing rate of primary end point in both age groups. However, this benefit of warfarin appeared to be offset by a higher rate of intracranial hemorrhage compared to aspirin, especially in patients aged more than 75 years. By contrast, several other contemporary trials of warfarin in patients with atrial fibrillation (BAATAF, CAFA, and SPINAF) which targeted lower intensity of anticoagulation (INR < 3) reported significantly lower rates of intracranial hemorrhage compared to SPAF-II, despite significant reduction in primary end point compared to placebo (BAATAF Investigators 1990; Connolly et al. 1991; Ezekowitz et al. 1992). These studies lead to the SPAF-III trial in which patients at high risk for thromboembolic complications were randomized to warfarin dose adjusted to INR 2–3 versus fixed low-dose warfarin (initially adjusted to INR 1.2–1.5) plus aspirin 325 mg daily (SPAF Investigators 1996). At end of follow-up, patients treated with dose-adjusted warfarin to INR 2–3 had significantly lower rates of primary end point compared to aspirin + low-dose warfarin (1.9 %/year versus 7.9 %/year, respectively) with no significant difference in major bleeding or intracranial hemorrhage. Importantly, there does appear to be a lower limit to this effect. INR values <2.0 do not appear to confer protection against stroke.

Several meta-analyses including all of the above studies and others have consistently shown that dose-adjusted warfarin is far more efficacious than aspirin in reducing thromboembolic complications as well as mortality in patients with atrial fibrillation (Hart et al. 1999; Cooper et al. 2006). In a 2007 meta-analysis by Hart et al., which included 29 trials with over 28,000 patients, dose-adjusted warfarin was found to produce significantly greater reduction in stroke compared to aspirin (39 % relative risk reduction) (Hart et al. 2007). Rate of major bleeding including intracranial hemorrhage did appear to be higher with dose-adjusted warfarin compared to aspirin, but the absolute risk increase was small (0.2 %/year) (Hart et al. 2007).

Aspirin Monotherapy

Anticoagulant therapy forms the mainstay of treatment in the vast majority of patients with atrial fibrillation based on its clear superiority in efficacy compared to aspirin. However, the value of aspirin as an alternative to anticoagulant therapy in patients with low risk of embolic complications or high risk of bleeding has been debated. Unfortunately, the benefit of aspirin compared even to placebo is also not clear. While several studies (particularly SPAF-I) have suggested a significant reduction in embolic events with aspirin, the 2007 meta-analysis by Hart et al. found that the 22 % reduction in events with aspirin compared to placebo was not statistically significant (Hart et al. 2007). In a meta-analysis of three trials enrolling 1965 patients with atrial fibrillation without prior stroke comparing aspirin at doses ranging from 75 to 325 mg daily to placebo, there was no difference in stroke or all-cause mortality (Aguilar and Hart 2005). Aspirin did reduce the combined end point of stroke, myocardial infarction, and cardiovascular mortality compared to placebo, though this was driven primarily by sensitivity analysis of a fourth trial (SAFT) in which aspirin therapy was combined with warfarin (Aguilar and Hart 2005; Edvardsson et al. 2003). Similarly, the Japanese Atrial Fibrillation Study which randomized 871 patients with atrial fibrillation to aspirin at 150–200 mg daily or no treatment showed that aspirin did not significantly reduce combined end point of stroke, TIA, or cardiovascular death but marginally increased risk of bleeding (Sato et al. 2006).

In summary, in patients with atrial fibrillation who cannot be treated with warfarin, the value of aspirin as an alternative to reduce thromboembolic complications is not clear. Furthermore, several recent studies have shown that patients who are not candidates for warfarin may be more appropriately treated with one of the newer oral anticoagulants instead of aspirin. For example, the AVERROES trial which randomized 5599 patients with atrial fibrillation who were unsuitable for warfarin therapy to apixaban 5 mg twice daily or aspirin 81 mg to 324 mg daily was terminated early due to clear benefit in favor of apixaban (Eikelboom et al. 2010). At mean follow-up of 1.1 years, rate of stroke or systemic embolism was significantly lower in patients receiving apixaban (1.3 %/year versus 3.75 %/year) while rate of major bleeding was no different (1.4 %/year versus 1.2 %/year).

Dual Antiplatelet Therapy with Aspirin and Clopidogrel

While multiple studies showed the superiority of warfarin to aspirin monotherapy in patients with atrial fibrillation, the relative efficacy of warfarin compared to dual antiplatelet therapy with aspirin and clopidogrel remained unanswered

until the ACTIVE W trial (Connolly et al. 2006). This study randomized 6706 patients with atrial fibrillation to dose-adjusted warfarin with INR 2.0–3.0 or clopidogrel 75 mg daily plus aspirin 75–100 mg daily. After mean follow-up of 1.3 years, the study was terminated early due to clear evidence of superiority of warfarin therapy with significantly lower composite outcome of stroke, systemic embolism, myocardial infarction, or vascular death (3.93 %/year versus 5.60 %/year). Severe or fatal bleeding was similar between the two groups, but minor bleeding was significantly higher in patients on dual antiplatelet therapy.

In patients deemed unsuitable for warfarin, the benefit of dual antiplatelet therapy with aspirin and clopidogrel in comparison to aspirin monotherapy was evaluated in the ACTIVE A trial (ACTIVE Investigators 2009). This study randomized 7554 patients with atrial fibrillation to aspirin 75–100 mg daily and clopidogrel 75 mg daily or aspirin alone. After mean of 3.6 years of follow-up, dual antiplatelet therapy was associated with significantly lower composite outcome of stroke, systemic embolism, myocardial infarction, or vascular death (6.8 %/year versus 7.6 %/year) compared to aspirin, but this benefit was offset by a significantly higher rate of major bleeding (2.0 %/year versus 1.3 %/year). However, these results need to be viewed in light of the observation that approximately one third of patients in this trial were ultimately treated with an oral anticoagulant after study withdrawal.

In summary, dual antiplatelet therapy does not seem to improve outcomes in patients with atrial fibrillation compared to aspirin alone and continues to be inferior to anticoagulant therapy. The question of managing patients with atrial fibrillation and stable coronary artery disease remains unanswered. Intuitively, one would expect that oral anticoagulant therapy would be needed to treat the atrial fibrillation, as demonstrated in the trials mentioned above, while antiplatelet therapy would be needed to manage the coronary artery disease. However, data to date have not confirmed this assumption. Based on results from a large Danish registry of atrial fibrillation, adding either aspirin or thienopyridine on top of vitamin K antagonist treatment failed to alter the risk of recurrent coronary events, while the risk of bleeding was increased by 50–84 % (Lamberts et al. 2014).

Triple Antithrombotic Therapy with Dual Antiplatelet Therapy plus an Anticoagulant

The appropriate antithrombotic regimen in patients with atrial fibrillation who also have an acute coronary syndrome or recent stent placement is controversial. The issue becomes more considerably complex among patients with acute coronary syndromes or recent intracoronary stent placement who also have requirements for oral anticoagulants. This population comprises between 5 and 10 % of patients receiving stents. The requirement for three antithrombotic drugs places such patients at elevated risk for hemorrhage. Several studies have indicated that oral anticoagulants are underused in stented patients with atrial fibrillation and that among patients in whom they are used, there is little relation between the perceived bleeding risk and the decision to use oral anticoagulants (Bernard et al. 2013; Ruiz-Nodar et al. 2012). A Danish registry indicated that the lowest bleeding risk and the lowest rate of thrombotic coronary events occurred among patients treated with a combination of oral anticoagulants and clopidogrel without aspirin rather than three drugs (Lamberts et al. 2013). A small randomized trial (WOEST) randomized 573 patients taking an oral anticoagulant to receive either aspirin plus clopidogrel or clopidogrel alone without aspirin for 1 year after stent placement. After 1 year, both the risk of bleeding and that of a composite end point of death, myocardial infarction, stroke, stent thrombosis, or target vessel revascularization were lower in the group treated with double therapy compared to those receiving triple therapy (Dewilde et al. 2013). A second trial (ISAR-TRIPLE) randomized patients who had been on an oral anticoagulant for at least 1 year to either 6 months of triple therapy or 6 weeks of triple therapy followed by therapy with the oral anticoagulant and clopidogrel without aspirin. At the end of 9 months, the rates of ischemic events as well as major bleeding (the primary outcome variable) were identical, although minor bleeding was more commonly seen in the group receiving triple therapy (Fiedler et al. 2015).

Take Home Messages

- Multiple randomized trials and meta-analyses have shown that aspirin reduces cardiovascular events in patients with established atherosclerotic vascular disease, especially in the setting of previous revascularization.
- The value of aspirin for primary prevention of cardiovascular events is unclear. Meta-analyses show that the benefit of aspirin in reducing ischemic events in low-risk patients may be offset by increased risk of bleeding. Ongoing trials aim to evaluate the benefit of aspirin for primary prevention in patients at moderate to high risk of atherosclerotic disease.
- Clopidogrel monotherapy has been shown to be as efficacious as aspirin for secondary prevention of cardiovascular events and therefore can be used as

(continued)

alternative to aspirin in patients with aspirin allergy.

- Dual antiplatelet therapy with aspirin and $P2Y_{12}$ inhibitors has been shown to be superior to aspirin monotherapy for prevention of ischemic events after percutaneous coronary intervention albeit with an increased risk of bleeding.
- The combination of aspirin and prasugrel was found to be superior to aspirin and clopidogrel in reducing ischemic events after percutaneous coronary intervention in the setting of acute coronary syndrome but with an increased risk of bleeding.
- The combination of aspirin and ticagrelor has been found to be superior to aspirin and clopidogrel in reducing cardiovascular death, myocardial infarction, and stroke after percutaneous coronary intervention both in the setting of acute coronary syndrome and stable coronary artery disease.
- The optimal duration of dual antiplatelet therapy after percutaneous coronary intervention is controversial and ultimately likely to depend on patient-specific factors that determine risk of ischemic as well as bleeding events.
- Antiplatelet monotherapy is clearly inferior to anticoagulant therapy in reducing ischemic events in patients with atrial fibrillation.
- Even dual antiplatelet therapy with aspirin and clopidogrel is inferior to anticoagulant therapy in reducing ischemic events in patients with atrial fibrillation.

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Antiplatelet Therapy in Cerebrovascular Disorders

Ilana Spokoyny and Gregory Albers

Abstract

Stroke is a preventable public health problem, with widespread effects. Antithrombotic medications are the mainstay of both primary and secondary stroke prevention. This chapter discusses the role of antiplatelet medications such as aspirin, clopidogrel, and dipyridamole in the acute treatment, primary prevention, and secondary prevention of stroke. For acute treatment, the current recommendations are early treatment with aspirin alone for acute ischemic stroke patients who are not receiving thrombolysis. For primary prevention, the recommendations do not generally recommend any antiplatelet unless the patient has atrial fibrillation, in which case anticoagulation is favored. For secondary prevention, the current data favors the use of an antiplatelet agent, either aspirin, clopidogrel, or the combination aspirin-dipyridamole. There is ongoing research into the role of combination aspirin plus clopidogrel, as this may be beneficial in certain subgroups of non-cardioembolic stroke patients. There is also ongoing research on novel antiplatelet agents, with the aim of decreasing recurrent stroke rates as well as bleeding events. This chapter provides a summary of current evidence and recommendations and addresses future possibilities for antiplatelet treatment for ischemic stroke.

Stroke is a worldwide health epidemic with significant public health consequences. Efforts at reducing the burden have focused on both treatment as well as prevention of recurrent events. The majority of strokes are ischemic, caused by insufficient blood flow to an area of brain. The optimal strategy for prevention of recurrent ischemic stroke is dependent on etiology. Decreased blood flow can be caused by emboli from the heart or more proximal arteries, in situ thrombosis of atherosclerotic arteries, global hypoperfusion causing watershed ischemia, or venous clots traveling through a PFO causing a paradoxical stroke. Risk factors for stroke include atrial fibrillation, which predisposes to cardioembolic strokes, as well as vascular risk factors such as hypertension, hyperlipidemia, smoking, and diabetes. Additionally, intrinsic hypercoagulable states place patients at increased risk of both venous thromboembolism and arterial stroke. The prevention strategies are tailored to the etiology of the ischemia, and for this reason, a comprehensive workup is recommended for all patients who suffer a stroke.

Antiplatelet agents have a role in the treatment of acute stroke as well as both the primary and secondary prevention of stroke. Anticoagulation is the treatment of choice for cardioembolic stroke (van Walraven et al. 2002), and it was historically regarded as the best treatment for some subtypes of non-cardioembolic ischemic stroke as well. However, randomized trials of anticoagulation versus antiplatelet therapy for non-cardioembolic stroke subtypes failed to demonstrate superiority of anticoagulation;

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therefore, antiplatelet agents have become the standard antithrombotic approach for secondary prevention of non-cardioembolic stroke. The WARSS trial (Mohr et al. 2001) compared aspirin (325 mg daily) to warfarin dosed to a target INR of 1.4–2.8 among patients with non-cardioembolic ischemic stroke and found no difference in the rates of ischemic stroke, death, or major bleeding between the two groups. The WASID trial (Chimowitz et al. 2005) compared aspirin (1300 mg daily) to warfarin dosed to a target INR of 2–3, among patients with TIA or mild stroke with symptomatic large vessel intracranial artery stenosis. The study was terminated early due to safety concerns in the warfarin arm. There was no difference between the two groups in the combined rate of ischemic stroke, cerebral hemorrhage, or vascular death. There was, however, a significantly decreased rate of death in the aspirin group (4.3 vs. 9.7 %, HR 0.46, 95 % CI 0.23–0.90) and a significantly lower rate of major bleeding (3.2 % vs. 8.3 %, HR 0.39, 95 % CI 0.18–0.84).

In this chapter, we summarize the data on the safety and effectiveness of antiplatelet agents for treatment and prevention of ischemic stroke.

Acute Stroke Treatment with Antiplatelet Agents

The risk of recurrent stroke in the acute period is highest in the first few days after stroke or transient ischemic attack (TIA) and is estimated to be between 3 and 10 % at 2 days (Mozaffarian et al. 2015). Acute treatment of stroke with antiplatelet agents does not reverse the symptoms of stroke, but rather is beneficial due to a reduction in the risk of recurrent stroke in the acute period (Bansal et al. 2013).

Aspirin

The acute use of aspirin for acute ischemic stroke has been studied in two large trials, the Chinese Acute Stroke Trial (CAST) (CAST (Chinese Acute Stroke Trial) Collaborative Group 1997) and the International Stroke Trial (IST) (International Stroke Trial Collaborative Group 1997). A meta-analysis (Sandercock et al. 2014) of the data from these trials and one other (Group 1995) (MAST-I) included patients treated within 48 h with ASA 160–300 mg daily for a duration of 2–4 weeks and demonstrated a 23 % odds reduction of recurrent ischemic stroke in the aspirin-treated patients. For every 1000 people treated with aspirin, seven people would avoid recurrent ischemic stroke, with a number needed to treat (NNT) of 140. There was also a reduction in death or dependency at the end of follow-up. Subgroup analyses did not show a difference in effect among different ages, genders, or ischemic stroke etiologies. Antiplatelet therapy did increase the odds of symptomatic intracranial hemorrhage by 23 %. For every 1000 people treated with aspirin, two would have a symptomatic intracranial hemorrhage, with a number needed to harm (NNH) of 574. Overall, the benefits of aspirin outweigh the risks in the early treatment of acute ischemic stroke, and so the early administration is recommended by multiple society guidelines (Lansberg et al. 2012; Jauch et al. 2013).

Ticagrelor

The SOCRATES clinical trial (Johnston et al. 2016) recently compared the effectiveness of early initiation of aspirin versus ticagrelor in acute ischemic stroke. This large trial included 13,199 patients with a non-severe ischemic stroke or high-risk TIA who had not received IV or IA thrombolysis and were not considered to have had a cardioembolic stroke. Patients were randomized within 24 h after symptom onset, to receive either ticagrelor 90 mg daily (180 mg loading dose on day 1) or aspirin 100 mg daily (300 mg on day 1) for 90 days. The primary end point was the time to the occurrence of stroke, myocardial infarction, or death within 90 days. Based on this primary end point, ticagrelor was not found to be superior to aspirin. The secondary end point was the time of first occurrence of any ischemic stroke, and ticagrelor showed a benefit over aspirin but with a nominal *p*-value because of the negative primary end point. In predefined exploratory analyses, there were indications that ticagrelor may be more effective at reducing ischemic stroke and all stroke. Additionally, ticagrelor seemed to most benefit those patients who were treated within 12 h of symptom onset and those who had been previously taking aspirin. A future direction of study may be aspirin plus ticagrelor within 12 h of stroke onset.

Combination Antiplatelet Therapy

There is ongoing research into the role of early initiation of dual antiplatelet agents after ischemic stroke and TIA. The CHANCE trial studied the combination of aspirin and clopidogrel in a Chinese population treated within 24 h of high-risk TIA or minor ischemic stroke (Wang et al. 2013). The authors compared aspirin 75 mg plus clopidogrel 75 mg for 21 days followed by clopidogrel alone for 90 days to placebo plus aspirin 75 mg for 90 days and demonstrated a significant decrease in the rate of stroke at 90 days. This

study included Chinese patients with high rates of severe intracranial atherosclerosis and was limited to mild stroke or high-risk TIA. Risk factor control (treatment of hypertension, diabetes, and hyperlipidemia) was suboptimal during the study. The results may not be generalizable to non-Chinese patients nor to those with moderate or severe stroke.

The EARLY trial compared treatment within 24 h with aspirin plus dipyridamole to aspirin alone for the first 7 days and then treated all patients with the combination therapy (Dengler et al. 2010). No difference in good outcome was found between the groups at 90 days. The FASTER trial compared early treatment (within 24 h) with aspirin plus clopidogrel to aspirin alone and was terminated early due to slow enrollment, but at 90 days showed no difference in stroke incidence between groups (Kennedy et al. 2007). A meta-analysis combined these studies and patients from 11 other studies who were treated with antiplatelet agents within 72 h, for a total of approximately 9000 patients (Wong et al. 2013). Dual antiplatelet therapy (which varied between trials and included aspirin plus clopidogrel, aspirin plus dipyridamole, and aspirin plus cilostazol) was associated with a 31 % decrease in recurrent stroke, without a significant increase in major bleeding. The results are limited in applicability by the variability in patient population and treatments, as well as the methodology of the trials included.

There are ongoing randomized trials evaluating the benefit of dual antiplatelet therapy compared to aspirin alone (POINT clinical trial; TARDIS clinical trial). In general, current recommendations favor early treatment with aspirin alone for acute ischemic stroke patients who are not receiving thrombolysis; there is insufficient evidence to support the use of alternative antiplatelet agents or dual antiplatelet therapy.

Primary Prevention of Ischemic Stroke

Data on primary stroke prevention with antiplatelet agents have largely come from larger studies which included cardiovascular end points such as myocardial infarction and death from any cardiovascular cause. Because the incidence of stroke is substantially lower in primary prevention studies, absolute risk reductions are small, and even a small increased risk of hemorrhage can neutralize the benefits for prevention of ischemic events. In primary prevention, aspirin has been shown to prevent MI, predominantly in men. Primary prevention of stroke has been demonstrated with aspirin in women with stroke risk factors.

Aspirin

There have been six large randomized trials evaluating the benefits of aspirin in primary prevention of cardiovascular events (Steering Committee of the Physicians' Health Study Research Group 1989; Iso et al. 1999; Collaborative Group of the Primary Prevention Project 2001; ETDRS Investigators 1992; Hansson et al. 1998; Peto et al. 1988). A meta-analysis of these studies showed a benefit of aspirin for MI but not for stroke or mortality (Bartolucci and Howard 2006). Sex differences have been identified with regard to primary prevention—two studies have shown a reduction in ischemic stroke among women taking aspirin for primary prevention (Berger et al. 2006; Ridker et al. 2005). The Antithrombotic Trialists' Collaboration published a large meta-analysis of six studies which included 95,000 patients, comparing antiplatelet agents (mostly aspirin) to placebo in patients with vascular risk factors, and found that primary prevention with aspirin reduced the incidence of serious vascular events by 12 %, but this was driven largely by a reduction in nonfatal MI. The overall effect on stroke was not significant (Baigent et al. 2009).

Clopidogrel

The CHARISMA trial compared combination aspirin and clopidogrel to aspirin alone. Subgroup analysis of patients with vascular risk factors but no prior ischemic event showed that the combination therapy was less effective than aspirin alone at reducing the rate of MI, stroke, or cardiovascular death (Bhatt et al. 2006).

Secondary Prevention of Ischemic Stroke

Antiplatelet therapy is the standard of care for secondary prevention of non-cardioembolic ischemic stroke. The most frequently studied agent has been aspirin; however, a number of trials have focused on either comparing aspirin with an alternative antiplatelet agent or testing a single antiplatelet agent against a combination of aspirin and an alternative antiplatelet agent.

Aspirin

The Antithrombotic Trialists' Collaboration analyzed 16 randomized controlled secondary prevention trials

comprising 17,000 patients and reported that aspirin therapy for secondary prevention reduced ischemic stroke by 22 % compared with placebo and was associated with a nonsignificant increase in hemorrhagic stroke (Baigent et al. 2009). The benefit was independent of age, sex, diabetes, or hypertension. Algra et al. published a large meta-analysis that pooled data from 11 randomized controlled trials comparing aspirin to control in approximately 9500 patients (Algra and van Gijn 1999). Long-term aspirin therapy reduced the risk of all serious vascular events by 13 %, which was mostly driven by a decrease in nonfatal stroke. The dose of aspirin varied between trials, but there was no evidence that higher doses offered better protection. A systematic review reported an increased risk of GI bleeding with doses of aspirin higher than 81 mg (Campbell et al. 2007). However, a large meta-analysis did not show a difference in bleeding rates comparing 75–162 mg to 162–325 mg daily of aspirin (McQuaid and Laine 2006). The 2002 Antithrombotic Trialists' Collaboration reviewed 195 secondary prevention trials and found no differences in effectiveness comparing doses of 75–150 mg daily versus 150–325 mg daily (Antithrombotic Trialists' Collaboration 2002).

Aspirin Atrial Fibrillation

For secondary prevention of stroke in patients with atrial fibrillation, aspirin is significantly less effective than oral anticoagulation. An individual patient meta-analysis of six trials demonstrated that patients treated with warfarin versus aspirin were 45 % less likely (HR 0.55) to have an ischemic stroke, with a small increase of major bleeding (van Walraven et al. 2002).

Clopidogrel

There is fairly limited data comparing clopidogrel to aspirin or placebo. The CAPRIE trial compared clopidogrel 75 mg daily versus aspirin 325 mg daily among patients with recent stroke, MI, or peripheral artery disease to determine which medication was more efficacious at preventing a composite end point of stroke, MI, or vascular death (CAPRIE Steering Committee 1996). Clopidogrel therapy conferred a relative risk reduction of 8.7 % compared with aspirin therapy. Aspirin-treated patients also had a significantly higher rate of GI bleeding. However, most of the benefit of clopidogrel was observed in patients with peripheral artery disease, and analysis of the stroke subgroup (>6300 patients) did not demonstrate a statistically significant benefit of clopidogrel over aspirin.

Combination Antiplatelet Therapy

Clopidogrel plus Aspirin

The MATCH trial compared clopidogrel plus aspirin to clopidogrel alone for the secondary prevention of TIA or stroke in high-risk patients (Diener et al. 2004). Combination therapy did not reduce the rate of recurrent stroke or TIA, or significant vascular events, but was associated with a significant increase in major bleeding complications. The combination of aspirin and clopidogrel was again tested in the CHARISMA trial (Bhatt et al. 2006). This trial evaluated both primary and secondary prevention of cardiovascular and cerebrovascular disease. CHARISMA compared the combination therapy to aspirin alone, rather than to clopidogrel monotherapy. Once again, combination therapy demonstrated no reduction in the risk of composite end point (MI, stroke, or cardiovascular death) while imposing a significantly increased risk of moderate bleeding and a nonsignificant increase in severe bleeding. Based on the results of these trials, the use of combination clopidogrel plus aspirin is not generally recommended for long-term secondary stroke prevention. The combination may have utility for short-term use, for patients with specific stroke subtypes that remain to be clarified (see CHANCE study discussed above and SAMMPRIS discussed below).

Clopidogrel plus Aspirin: Lacunar Stroke

The SPS3 trial evaluated patients with lacunar stroke and compared the combination of aspirin and clopidogrel to aspirin alone (Benavente et al. 2012). However, the study was terminated early due to increased bleeding events and mortality in the dual antiplatelet group. Furthermore, no reduction in recurrent stroke was observed. The MATCH study (Diener et al. 2004) had a large subset of patients with lacunar stroke, and the results of these studies taken together argue against the use of dual antiplatelet therapy for long-term stroke prevention among patients with small-vessel subcortical stroke.

Clopidogrel plus Aspirin: Large Artery Intracranial Atherosclerosis

The SAMMPRIS trial compared angioplasty and stenting plus aggressive medical management to aggressive medical management alone in patients with symptomatic large artery intracranial atherosclerosis (Chimowitz et al. 2011). The medical management consisted of 90 days of dual antiplatelet therapy (aspirin and clopidogrel) along with a high-dose statin. The study demonstrated a lower rate of stroke and death in the medical arm compared to the

intervention arm, as well as compared to historical controls, demonstrating that the short-term use of combination aspirin plus clopidogrel in patients with large artery intracranial atherosclerosis was associated with favorable outcomes.

Clopidogrel plus Aspirin: Embolic Stroke from Aortic Plaque

The ARCH trial compared combination aspirin plus clopidogrel to warfarin with embolism due to aortic plaque and did not show a difference between these two therapies but was terminated early due to slow enrollment (Amarenco et al. 2014).

Clopidogrel plus Aspirin: Embolic Stroke from Atrial Fibrillation

The ACTIVE W (Connolly et al. 2006) and ACTIVE A (Connolly et al. 2009) trials evaluated the safety and efficacy of dual antiplatelet therapy in patients with atrial fibrillation. ACTIVE W compared clopidogrel plus aspirin to warfarin, and ACTIVE A compared clopidogrel plus aspirin to aspirin alone. ACTIVE A included only patients who were not candidates for anticoagulation. The ACTIVE W trial was stopped early because warfarin significantly lowered the rate of the composite end point (stroke, MI, or cardiovascular death) compared to combined antiplatelet therapy. There was also a trend toward less major bleeding with warfarin. The ACTIVE A trial demonstrated that patients treated with clopidogrel plus aspirin had a significantly lower annual rate of the composite end point and that the majority of this effect came from the 27 % reduction in stroke. However, dual antiplatelet therapy had a significantly increased incidence of major bleeding compared to aspirin. Based on the ACTIVE A trial, dual antiplatelet therapy with aspirin plus clopidogrel is an alternative to aspirin monotherapy for secondary stroke prevention in patients with atrial fibrillation patients who are unable to be treated with oral anticoagulation. However, dual antiplatelet therapy and warfarin have similar bleeding rates, and the newer oral anticoagulants likely have lower bleeding risks; therefore, increased bleeding risk is not good reason for selecting dual antiplatelet therapy in a patient with atrial fibrillation.

Dipyridamole plus Aspirin

The ESPS-2 trial compared 200 mg extended-release dipyridamole (ER-DP) twice daily, 25 mg aspirin twice daily, combination aspirin 25 mg and 200 mg ER-DP twice daily, and placebo in a four-armed study (Diener et al. 1996). The combination therapy was the most efficacious at preventing recurrent events (OR 0.59 compared to placebo), followed by aspirin monotherapy (OR 0.81 compared to placebo) and then ER-DP monotherapy (OR 0.79 compared to placebo). The ESPRIT trial compared open-label aspirin plus or minus dipyridamole 200 mg twice daily (Halkes et al. 2006). The composite outcome of stroke, MI, major bleeding, or vascular death was reduced in the dual antiplatelet group by 20 % (HR 0.80) compared to aspirin alone.

Since there was evidence the aspirin plus ER-DP was superior to aspirin alone (ESPS-2 and ESPRIT) and some evidence that clopidogrel was superior to aspirin alone (CAPRIE), the PRoFESS trial (Sacco et al. 2008) sought to compare these two treatments. The trial randomized 20,000 patients to either aspirin/ER-DP (25/200 mg twice daily) or clopidogrel 75 mg daily. No difference was seen in either recurrent stroke or composite end point of stroke, MI, or vascular death. There was a difference in adverse effects—more patients discontinued aspirin/ER-DP due to headache; there was also a trend toward more bleeding on the combination therapy.

Novel Agents

Adjuncts to Acute Therapy

Some antiplatelet medications have been considered for use as adjunct therapy to acute stroke thrombolysis, specifically the glycoprotein IIb/IIIa receptor antagonists such as tirofiban and eptifibatide. These have been evaluated in Phase II trials and were shown to be safe, but Phase III trials are needed to determine their efficacy (Pancioli et al. 2008, 2013; Siebler et al. 2011).

Secondary Prevention

Other antiplatelet agents including ticagrelor, prasugrel, and cangrelor have been recently studied in cardiac patients (Wallentin et al. 2009; Wiviott et al. 2007). The SOCRATES trial (Johnston et al. 2016) failed to show a benefit of ticagrelor over aspirin in the prevention of a composite outcome, but the others are yet to be studied in cerebrovascular disease and represent potential future targets.

Summary and Recommendations

Nonfatal recurrent stroke (ischemic and hemorrhagic) in patients with non-cardioembolic stroke or TIA [Adapted from CHEST Guidelines (Lansberg et al. 2012)]

Comparison	No. of participants (studies) follow-up	Relative effect (95 % CI)	Anticipated absolute effects, time frame of 2 years	
No aspirin vs. aspirin	10,126 (11 studies) 1.5–6 years	RR, 0.81 (0.71–0.92)	**Risk with no aspirin**	**Risk difference with aspirin (95 % CI)**
			130 strokes per 1000	25 fewer strokes per 1000 (from 10 fewer to 38 fewer)
Aspirin vs. clopidogrel	6431 (1 study) 1.9 years	RR, 0.91 (0.78–1.07)	**Risk with aspirin**	**Risk difference with clopidogrel (95 % CI)**
			106 strokes per 1000	10 fewer strokes per 1000 (from 23 fewer to 7 more)
Aspirin vs. aspirin + dipyridamole	7659 (6 studies) 2.6 years	RR, 0.77 (0.67–0.89)	**Risk with aspirin**	**Risk difference with aspirin plus dipyridamole (95 % CI)**
			106 strokes per 1000	24 fewer strokes per 1000 (from 12 fewer to 35 fewer)
Clopidogrel vs. aspirin + dipyridamole	20,332 (1 study) 2.5 years	HR, 0.97 (0.88–1.07)	**Risk with clopidogrel**	**Risk difference with aspirin plus dipyridamole (95 % CI)**
			97 strokes per 1000	3 fewer strokes per 1000 (from 11 fewer to 6 more)
Clopidogrel vs. aspirin + clopidogrel	7599 (1 study) 18 months	RR, 0.95 (0.82–1.1)	**Risk with clopidogrel**	**Risk difference with aspirin plus clopidogrel (95 % CI)**
			97 strokes per 1000	5 fewer strokes per 1000 (from 17 fewer to 10 more)
Antiplatelet vs. oral anticoagulant	5400 (5 studies) 0–5 years	RR, 1.03 (0.88–1.22)	**Risk with antiplatelet**	**Risk difference with oral anticoagulation (95 % CI)**
			106 strokes per 1000	3 more strokes per 1000 (from 13 fewer to 23 more)

Take-Home Messages

- Antiplatelet agents are the standard antithrombotic treatment for secondary prevention of non-cardioembolic stroke.
- For the early treatment of acute ischemic stroke:
  - Aspirin is recommended.
  - There is insufficient evidence to support the use of alternative antiplatelet agents or dual antiplatelet therapy.
- For primary prevention of acute ischemic stroke:
  - The data is mixed and requires further investigation.
  - Aspirin may be effective at preventing strokes in women.
  - Dual antiplatelet therapy is not as safe or effective for primary prevention of vascular events as aspirin.
- For secondary prevention of acute ischemic stroke:
  - Aspirin reduced rates of recurrent ischemic stroke compared with placebo.
  - It is unclear whether clopidogrel outperforms aspirin for secondary prevention—the single study used a combined end point, and clopidogrel's better outcomes were not driven by stroke prevention.
  - There is no difference between the effectiveness of aspirin/ER-DP and clopidogrel, but aspirin/ER-DP had more side effects including headache and bleeding.
  - Use of combination aspirin plus clopidogrel is not recommended for long-term secondary stroke prevention.
  - Short-term use of combination aspirin plus clopidogrel in patients with large artery intracranial atherosclerosis may be beneficial.
  - Aspirin plus clopidogrel is a more effective alternative to aspirin monotherapy for secondary stroke prevention in patients with atrial fibrillation who are unable to be treated with oral anticoagulation. However, dual antiplatelet therapy and warfarin have similar bleeding rates.

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Antiplatelet Therapy in Peripheral Artery Disease

Francesco Violi, Stefania Basili, Jeffrey S. Berger, and William R. Hiatt

Abstract

Peripheral artery disease (PAD) is a problem of substantial public health importance and an important hallmark of generalized atherosclerosis involving, in particular, coronary and cerebral circulation. Despite the high prevalence of PAD, in particular in older population, this disease is underdiagnosed because it often presents with atypical symptoms or no ischemic symptoms. Ankle-brachial index (ABI) is a simple, inexpensive noninvasive test, which is widely accepted as a diagnostic test used to evaluate the presence of PAD. The ABI is also demonstrated to be useful in the assessment of vascular risk in asymptomatic and symptomatic patients being correlated with increased risk of coronary heart disease, stroke, and cardiovascular death. The risk factors for PAD are similar to the risk factors for atherosclerosis; thus, smoking should be stopped, and hypertension, diabetes mellitus, and dyslipidemia should be treated. In addition, antiplatelet therapy remains a key intervention to reduce cardiovascular risk in PAD.

In patients with PAD who have clinical evidence of concomitant coronary or cerebrovascular disease, aspirin or clopidogrel would be first-line. However, the data supporting the use of antiplatelet drugs in patients with PAD, who do not have a history of other cardiovascular disorders, are still inconclusive and further study is necessary to explore this issue.

Epidemiology and Classification of PAD

Peripheral artery disease (PAD) is a term that relates to atherosclerosis and narrowing of the arteries in the lower extremities. The spectrum of PAD encompasses acute limb ischemia and chronic limb ischemia, which is further divided into asymptomatic, claudication, and critical limb ischemia (Fig. 1) (Hiatt et al. 2008; McDermott 2015). PAD is a problem of substantial public health importance. It has been estimated that approximately 27 million persons in North America and Europe are afflicted with PAD (Rosamond et al. 2008; Belch et al. 2003). The prevalence of PAD is approximately 12 % of the adult population (Fig. 2), with men being affected slightly more than women (Criqui et al. 1985; Kannel and McGee 1985). PAD is increasingly prevalent in the aging population. Findings from a national cross-sectional survey of PAD Awareness, Risk, and Treatment: New Resources for Survival (PARTNERS) found that PAD afflicts 29 % of patients who are age >70 years or have risk factors of diabetes or smoking (Hirsch et al. 2001).

PAD is an important hallmark of generalized atherosclerosis involving, in particular, coronary and cerebral circulation (Bhatt et al. 2006). Thus, patients with PAD are at high risk of suffering from myocardial infarction, stroke, and vascular death with an annual incidence of about 5 % (Steg et al. 2007). Nevertheless, perception of the specific

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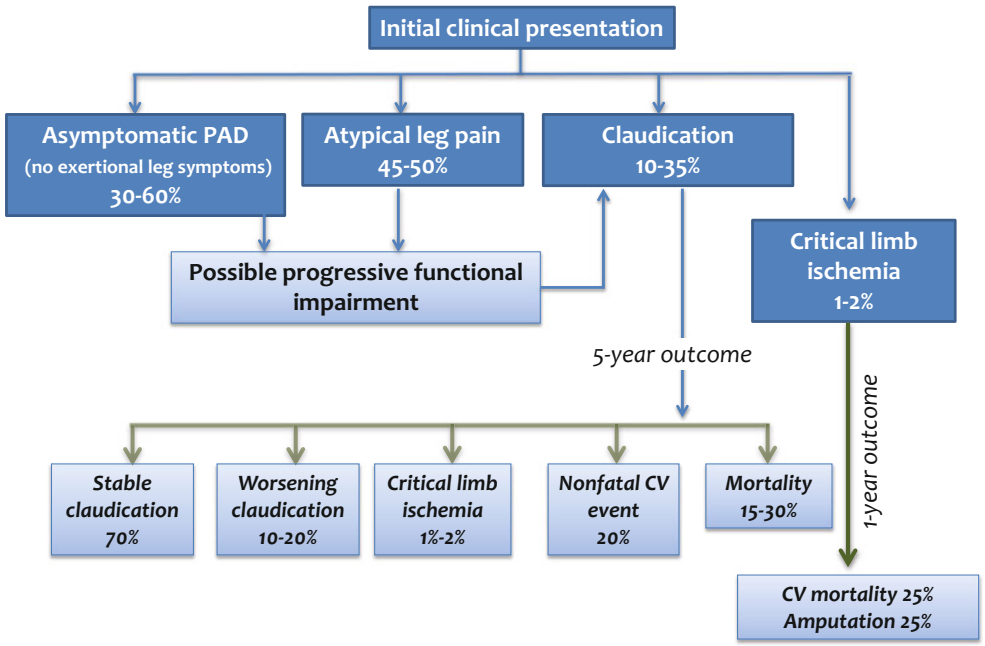

Fig. 1 Natural history of patients with peripheral artery disease

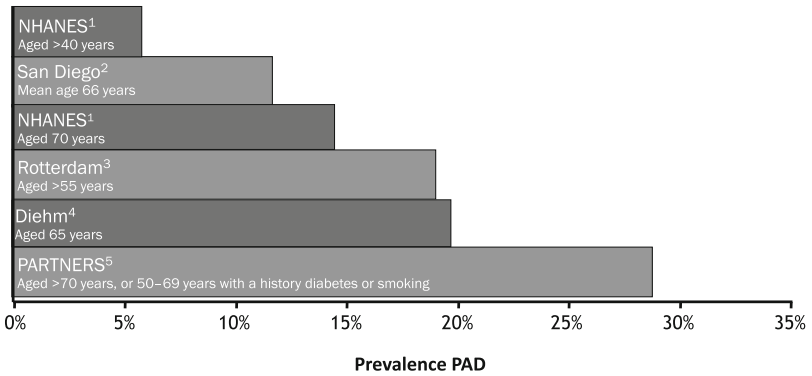

Fig. 2 Prevalence of peripheral artery disease. *NHANES* National Health and Nutrition Examination Study, *PARTNERS* PAD Awareness, Risk, and Treatment: New Resources for Survival [program]. (1) Circulation. 2004;110:738–743. (2) Circulation. 1985;71:510–515. (3) Atherosclerosis. 2004;172:95–105. (4) Arterioscler Thromb Vasc Biol. 1998;18:185–192. (5) JAMA. 2001;286:1317–1324

risks associated with PAD is generally poor compared with other atherothrombotic settings, such as coronary artery disease or cerebrovascular disease. In fact, PAD represents a growing problem for internists since it is not just a localized disease, but it has serious systemic complications (Stehouwer et al. 2009; Pande et al. 2011).

Despite the strikingly high prevalence of PAD, this disease is underdiagnosed because it often presents with atypical symptoms or no ischemic symptoms related to the legs at all as showed by the Walking and Leg Circulation Study (McDermott et al. 2002). Accordingly, asymptomatic disease may occur in up to 50 % of all patients with PAD (Hirsch et al. 2006) (Fig. 1). In the Rotterdam Study, there was a 19.1 % prevalence of PAD, but claudication was reported in only 6.3 % (Meijer et al. 1998).

Ankle-brachial index (ABI) is the ratio of the ankle to brachial systolic blood pressure. This simple, inexpensive noninvasive test is widely accepted as a diagnostic test used to evaluate the presence of lower extremity peripheral artery disease, defined by an ABI $\leq$ 0.90 in patients with symptoms of claudication or rest ischemia (Fig. 3) (Aboyans et al. 2012; Norgren et al. 2007a, b; Criqui et al. 2008).

The ABI is also demonstrated to be useful in the assessment of vascular risk in asymptomatic and symptomatic patients. Screening for PAD in asymptomatic individuals should be considered in terms of cardiovascular risk and not merely of limb outcomes (Beckman et al. 2006).

Numerous studies have demonstrated that an abnormal ABI correlates with a significantly increased risk of vascular

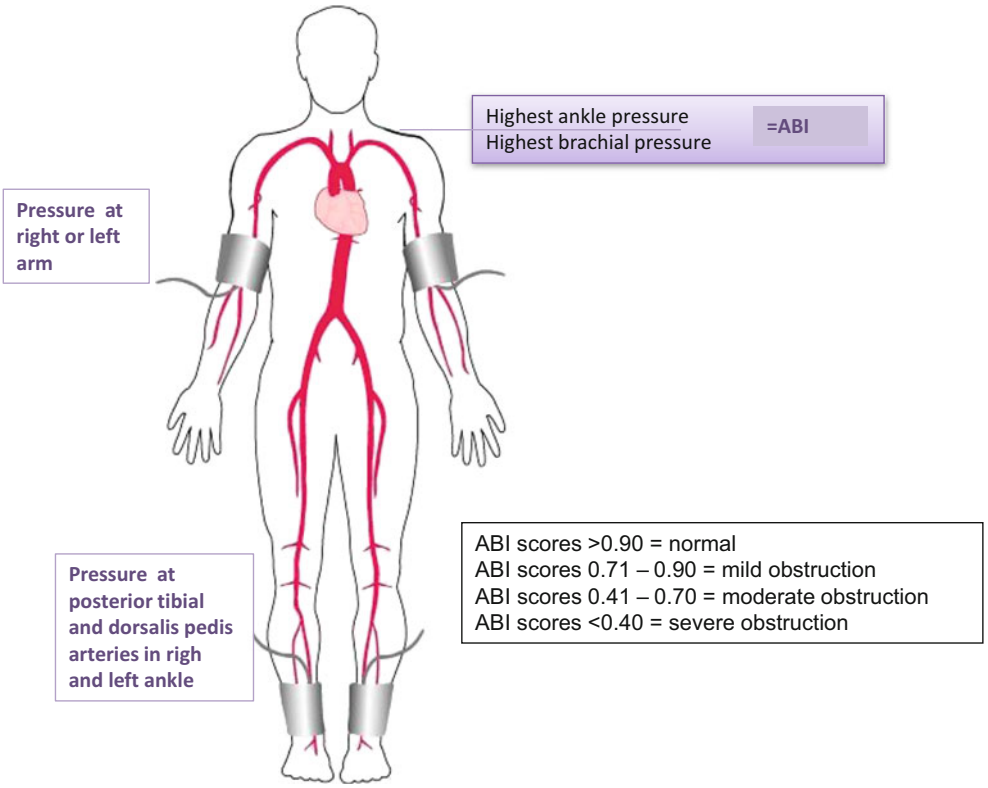

Fig. 3 Ankle-brachial index

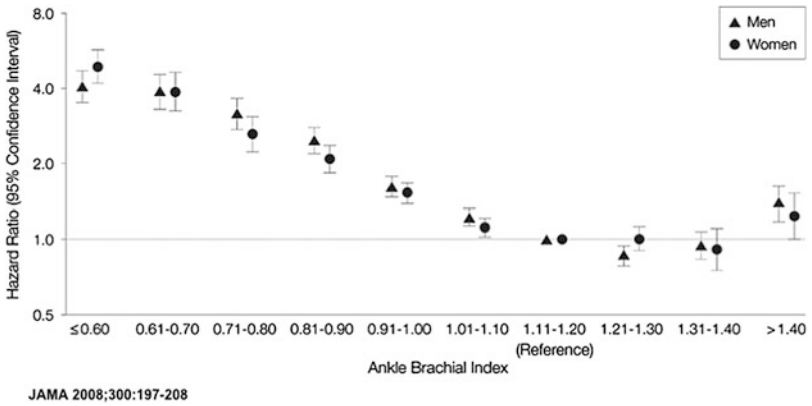

Fig. 4 Hazard ratios for total mortality in men and women by ankle-brachial index at baseline for all studies combined in the ABI collaboration. Pending permission from JAMA

events (Fowkes 2008; Doobay and Anand 2005; Resnick et al. 2004; O'Hare et al. 2006; McDermott et al. 2005).

However, an ABI $\leq$ 0.90 in population-based cohort studies seems to have high specificity but not enough sensitivity for predicting vascular events. Combining the population-based cohorts and the high-risk cohorts, the overall prevalence of ABI $\leq$ 0.90 is 13.2 % (Doobay and Anand 2005). Because of its low sensitivity (yet high specificity), the ABI cannot be used as a generic screening test. Rather, it must be used in a focused manner, choosing individuals for whom the yield of the test is expected to be higher. The sensitivity of a low ABI to predict all-cause mortality is much higher in the high-risk cohorts compared with the population-based cohorts (85.0 % vs. 31.2 %). The sensitivity and specificity of a low ABI to predict incident coronary heart diseases were 16.5 % and 92.7 %, for incident stroke 16.0 % and 92.2 %, and 41.0 % and 87.9 % for cardiovascular mortality, respectively. Furthermore, a meta-analysis of 16 population cohort studies was performed to determine if the ABI provides information on the risk of cardiovascular events and mortality independently of the Framingham Risk Score (FRS) and if it can improve risk prediction (Fowkes 2008). Including ABI in cardiovascular risk stratification using Framingham Risk Score would result in reclassification of the risk category and modification of treatment recommendations in approximately 19 % of men and 36 % of women (Fig. 4). In the hazard ratios (HRs) for death for different levels of ABI compared with an ABI

reference of 1.11–1.20 (low risk), a reverse J-shaped curve for both men and women was detected. For levels of ABI below 1.11, the HRs increased consistently with decreasing ABI as well as for an ABI of greater than 1.40 in both men and women. Conversely, no differences were found for levels of ABI from 1.11 to 1.40. In nearly all the studies in men, the HRs for total mortality were statistically significantly higher in individuals with an ABI of 0.90 or less compared with individuals with normal ABI values of 1.11–1.40. Likewise, significantly increased HRs were found in men and in women both for cardiovascular mortality and for major coronary events. Adjustment of the HRs for individuals with an ABI of 0.90 or less relative to an ABI of 1.11–1.40 for FRS reduced the HRs that, however, were still significantly elevated. Thus, the authors concluded that ABI provided independent risk information compared with the FRS and, when combined with the FRS, low ABI was associated with approximately twice the 10-year total mortality, cardiovascular mortality, and major coronary event rate across all Framingham risk categories. Thus, screening for PAD in asymptomatic individuals should be considered in terms of cardiovascular risk and not merely of limb outcomes.

Management of PAD

The risk factors for PAD are similar to the risk factors for atherosclerosis elsewhere (Fig. 5); the most strongly associated with PAD, in addition to age, are cigarette smoking and diabetes mellitus (DM) (Norgren et al. 2007a, b; Criqui and Aboyans 2015; Aronow 2010). Thus, smoking should be stopped, and hypertension, diabetes mellitus, and dyslipidemia should be treated. In addition, antiplatelet therapy remains a key intervention to reduce cardiovascular risk in PAD. Exercise rehabilitation programs and cilostazol help to relieve symptoms and improve exercise performance (Aronow 2010).

Platelets play an important role in the process of atherothrombosis via release of molecules that are injurious for vascular walls and by precipitating thrombosis at the site of plaque injury. There is evidence indicating that platelets are activated in patients with PAD. Platelet activation as assessed by urinary excretion of 11-dehydro-TxB_2 has been studied in patients with PAD and matched controls (Davì et al. 1997). This study demonstrated enhanced values of urinary 11-dehydro-TxB_2 excretion in PAD patients compared with controls. Such differences, however, seemed to be attributable essentially to the coexistence of risk factors for atherosclerotic disease such as hypercholesterolemia, diabetes, or smoking. Thus, in PAD patients without such risk factors, urinary excretion of 11-dehydro-TxB_2 was similar to that in controls, suggesting that the risk factors rather than atherosclerotic disease, per se, are responsible for enhanced production of TxA_2. Analysis of other markers of

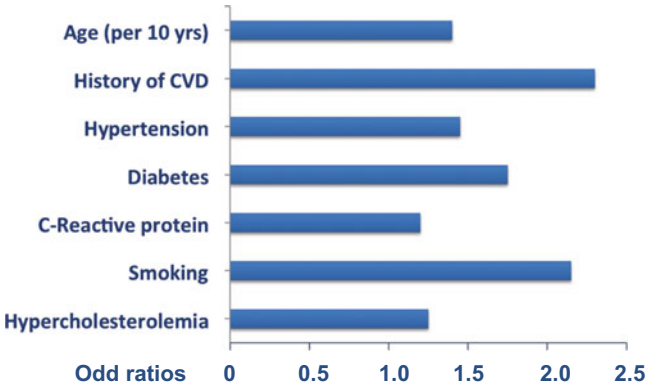

Fig. 5 Risk factors for peripheral artery disease

platelet activation confirmed the existence of platelet activation in PAD. In particular, a significant association between mean platelet volume (MPV) and PAD (as defined by a screening ABI < 0.90 even after multivariable adjustment) and high levels of soluble CD40 ligand and P-selectin have been detected in patients with PAD and critical leg ischemia (Berger et al. 2010; Blann et al. 2005). However, there was no relationship between the severity of arteriopathy, defined as the presence of resting pain, and platelet activation. Therefore, it is still unclear if platelet activation is a reflection of peripheral atherosclerosis or of the coexistence of risk factors such as diabetes, dyslipidemia, and smoking that activate, per se, platelet function.

Antiplatelet Drugs in PAD

Antiplatelet agents are a therapeutic cornerstone in the secondary prevention of cardiovascular events in patients with established atherosclerosis. The essential role of antiplatelet therapy has been well established in serial publications of the Antithrombotic Trialists' Collaboration (ATC) including over 130,000 patients and 50 trials (ATC 2002). This meta-analysis clearly demonstrates a cardioprotective benefit of antiplatelet therapy in a broad population of patients with clinical evidence of atherosclerosis. Specifically, this high-risk population has been defined as those with a history of myocardial infarction, acute coronary syndrome, transient ischemic attacks, or stroke, as well as coronary or carotid revascularization procedures. These are generally patients who have had ischemic events or interventions for symptomatic disease. In the subgroup of patients with stable peripheral arterial disease (PAD), similar benefits of antiplatelet therapy were observed. Thus, antiplatelet treatment was associated with a 23 % risk reduction of vascular events including MI, stroke, and vascular death in overall population with PAD. However, closer scrutiny of these data reveals that

the benefits in the PAD subgroup were largely driven by non-aspirin antiplatelet drugs including ticlopidine, clopidogrel, picotamide, and dipyridamole.

Aspirin and PAD

Aspirin is the most used antiplatelet drug to prevent cardiovascular events in patients with cardiovascular disease. Aspirin inhibits platelet COX1, thereby preventing the formation of thromboxane (Tx) A_2, a potent aggregating and vasoconstrictor molecule (FitzGerald 1991).

At variance with other circulatory territories such as coronary and cerebral trees, the effect of aspirin in peripheral atherosclerosis has been less well investigated, and results are inconclusive. The effect of aspirin has been examined in a post hoc analysis of the Physicians' Health Study where aspirin was ineffective in preventing claudication deterioration, while it seemed to reduce the incidence of peripheral surgical interventions (Goldhaber et al. 1992). However, these data are difficult to interpret due to the retrospective nature of the study. Therefore, evidence for any benefit of aspirin treatment in patients with PAD should be considered insufficient.

The American College of Chest Physicians (Alonso-Coello et al. 2012) practice guidelines recommended aspirin treatment (75–100 mg/day) in patients aged ≥50 years with asymptomatic PAD over no therapy (Grade 2B) for the primary prevention of cardiovascular events. For secondary prevention of cardiovascular disease in patients with symptomatic PAD (including patients before and after peripheral arterial bypass surgery or percutaneous transluminal angioplasty), long-term aspirin (75–100 mg/day) or clopidogrel (75 mg/day) (Grade 1A) were recommended. Moreover, 2011 ACCF/AHA Focused Update of the Guideline for the Management of Patients With Peripheral Artery Disease (Rooke et al. 2011) recommended aspirin, typically in daily doses of 75–325 mg, to reduce the risk of MI, stroke, or vascular death in individuals with symptomatic atherosclerotic lower extremity PAD (Level of evidence: B).

Three RCTs of antiplatelet therapy versus placebo for cardiovascular risk reduction among patients with PAD specifically examined the role of aspirin in PAD.

In the Critical Leg Ischaemia Prevention Study (CLIPS) (Catalano 2007), a study with aspirin in PAD anticipated randomizing 2000 patients, the enrollment was discontinued prematurely after including only 210 PAD patients. The reasons for stopping early were feasibility and not due to encountering any predefined stopping rule. Patients were randomized to placebo or 100 mg aspirin and followed up for 2 years. Throughout the study, major cardiovascular events, including fatal and nonfatal cerebral ischemia and myocardial infarction and critical leg ischemia, were monitored. During the follow-up, there were seven major cardiovascular events in the aspirin-treated group and 20 in the placebo-treated group, with a hazard ratio of 0.35, 95 % confidence interval (CI), 0.15–0.82. However, this benefit was entirely driven by a decrease in nonfatal events, as there were seven deaths on aspirin and four in patients randomized to the control group. Despite such interesting findings, the relatively few number of events and imbalance in deaths did not permit us to reach definite conclusions on the clinical efficacy of aspirin in the PAD population.

In the POPADAD study (Belch et al. 2008), Belch et al. investigated whether aspirin and antioxidants given together or separately can reduce MI and death in patients with diabetes and PAD. In their study, 1276 patients with diabetes and evidence of asymptomatic PAD (as determined by a lower-than-normal ankle-brachial pressure index of 0.99 or less, but no symptoms) over 40 years of age were randomized to receive either aspirin 100 mg or placebo, an antioxidant or placebo, or aspirin and an antioxidant or double placebo and followed over 8 years. There were two hierarchical composite primary end points: death from coronary heart disease or stroke, nonfatal MI or stroke, or amputation above the ankle for critical limb ischemia and death from CHD or stroke. Overall, the researchers found no benefit from either aspirin or antioxidant treatment. Patients in the aspirin groups had 116 primary events compared with 117 in the placebo group [hazard ratio (HR) 0.98 (95 % confidence interval, 0.76–1.26); $p = 0.86$]. There were 43 deaths from CHD or stroke in the aspirin group compared with 35 in the no-aspirin group [HR 1.23 (0.79–1.93); $p = 0.36$].

It is important to recognize that POPADAD is smaller than most of the other aspirin trials, with fewer events and that it is possible that small effects may be shown with larger trials continued for a longer time.

In 2010, the results of Aspirin for Asymptomatic Atherosclerosis (AAA) trial were published (Fowkes et al. 2010). The Aspirin for Asymptomatic Atherosclerosis (AAA) trial, conducted from April 1998 to October 2008, was an intention-to-treat, double-blind, randomized controlled trial of once-daily low-dose enteric-coated aspirin (100 mg) vs. placebo. It involved 28,980 men and women, aged 50–75 years, who were free of clinical cardiovascular disease, recruited from a community Scottish health registry. All recruited patients had an ABI screening test. Of those, 3350 with a low ABI (defined as an ABI value equal to or less than 0.95) entered the trial. The trial was designed for 80 % power to detect 25 % proportional risk reduction in events. Enrolled patients were randomly assigned in 1:1 ratio to low-dose aspirin (100 mg/daily) or to placebo groups. The primary end point of this study was a composite of fatal or nonfatal coronary event or stroke or revascularization. The follow-up had to be extended from the originally planned 5 years to 9.5 years. After a mean (SD) ;follow-up of 8.2 (1.6) years, aspirin was no more effective than placebo in reducing the primary end point (13.7 events per 1000 person-years in the aspirin group vs. 13.3 events

per 1000 person-years in the placebo group; HR, 1.03; 95 % CI, 0.84–1.27). Moreover, aspirin treatment had no significant effect on any of the secondary end points. Aspirin therapy was, however, associated with a nonsignificant increased risk of major hemorrhage (2.0 % vs. 1.2 %; HR = 1.71, 95 % confidence interval, 0.99–2.97). Intracranial hemorrhage occurred in 11 participants (including three fatal subarachnoid/subdural hemorrhages) in the aspirin group and seven in the placebo group. These results are similar to the findings of the aspirin primary prevention trials. Based on these findings, the authors concluded that in asymptomatic PAD patients, aspirin had no clinical benefit and may be harmful.

Additional findings were achieved by a meta-analysis performed by Berger et al. (2009) to assess the benefit of aspirin in treating both symptomatic and asymptomatic PAD patients including 18 prospective, randomized controlled trials of aspirin alone or in combination with other antiplatelet drugs. The meta-analysis was designed to test the null hypothesis that aspirin was not different from placebo or control in reducing the risk of the combined primary end point of nonfatal MI, nonfatal stroke, and cardiovascular death. Among 5269 participants included in the analysis, cardiovascular events were experienced in 251 (8.9 %) of 2823 patients taking aspirin (alone or with dipyridamole) and in 269 (11.0 %) of 2446 in the control group. The pooled RR reduction of 12 % in cardiovascular event rates was not statistically significant. Moreover, in the subset of 1516 participants taking aspirin monotherapy compared to control, aspirin was associated with a nonsignificant reduction in cardiovascular events [8.2 % vs. 9.6 %; RR = 0.75 (95 % CI, 0.48–1.18)] and in all-cause or cardiovascular mortality, MI, or major bleeding.

Almost 20 years ago, the US Food and Drug Administration (FDA) was asked to extend the labeling of aspirin to include PAD patients (FDA 1998). In its deliberations, the FDA could not find substantive evidence to support the role of aspirin for this indication. The implication of this deliberation was that patients with PAD who do not have overt clinical manifestations of atherosclerosis in other arterial beds might not be responsive to aspirin chemoprophylaxis.

With the absence of any new evidence, one should reserve aspirin therapy for PAD patients who have suffered coronary or cerebral ischemic events. This information could be of some importance in the design of additional placebo-controlled trials of aspirin in larger PAD populations.

Non-aspirin Drugs and PAD Drugs Inhibiting the Platelet Arachidonic Acid Pathway

Picotamide, a derivative of methoxyisophthalic acid, is an antiplatelet drug whose pharmacological properties consist in inhibiting both thromboxane A_2 receptors and TxA_2 synthase. As concentrations of the molecule needed to inhibit both pathways are almost equivalent (Modesti et al. 1989, 1994; Violi et al. 1988; Gresele et al. 1989; Berrettini et al. 1990; Cattaneo et al. 1991), picotamide may exert a dual pharmacological action in vivo and be potentially useful in various clinical settings characterized by atherosclerotic disease. Two prospective studies have been performed in PAD populations with different risks for cardiovascular events: the ADEP and DAVID trials.

The first large randomized trial (ADEP trial) on picotamide investigated its clinical usefulness in patients with PAD (Balsano and Violi 1993). For this study, 2304 patients were consecutively enrolled, allocated to either placebo or picotamide (300 mg tid), and followed up for 18 months. End points of the study were major events (i.e., cardiovascular death, myocardial infarction, stroke, or amputation) and minor events (unstable angina, transient ischemic attack, hypertension, renal failure, deterioration of PAD). The "intention-to-treat analysis" showed a risk reduction (18.9 %) in the combined end points, major plus minor events, in the picotamide group compared with the controls which, however, did not reach statistical significance. Conversely, "on-treatment analysis" showed a higher and statistically significant reduction (22.8 %) in the same end points. Side effects such as bleeding were almost identical in the two groups. As the authors suggested, the lack of any beneficial effects of picotamide against major events could have been related to the low occurrence of these events during the follow-up. This phenomenon may be related to a bias in patient selection, which excluded high-risk patients. The capacity of picotamide to prevent vascular complications was, however, magnified when claudicant patients affected by diabetes were taken into account. Thus, a sub-study of the ADEP trial retrospectively analyzed 438 diabetic patients and observed a risk reduction of 45.2 % of combined major and minor events in those treated with picotamide compared with those treated with placebo (Milani et al. 1996). On the basis of this post hoc analysis, another randomized trial (the DAVID trial) was specifically designed for diabetic patients with PAD (Neri Serneri et al. 2004). Thus, 1209 patients were enrolled and randomly assigned to picotamide (600 mg/bid) or aspirin (320 mg/day) and followed up for 2 years. The primary end point was overall mortality, and the secondary one was the combined incidence of death and major cardiovascular events. Mortality was significantly lower in picotamide-treated patients than in those treated with aspirin, showing a relative risk of reduction of 45 %. Furthermore, the incidence of gastrointestinal bleeding was much lower in the picotamide group than in the aspirin group. The secondary end point did not show any significant difference between the two populations, showing only a nonsignificant trend in favor of patients taking picotamide. As pointed out by the authors, a possible bias relative to the high proportion of patients

(about 20 % in each group) who discontinued the trial because of nonfatal events may have underestimated the real incidence of the secondary end points. Moreover, it is possible that the sample size of the study was insufficient to detect any difference in these end points between the two groups. Comparison of the results achieved by the ADEP and DAVID trials also raises the question as to whether the differences seen are dependent on the fact that TxA_2 production is more relevant for atherosclerotic progression in PAD patients with diabetes compared with PAD without diabetes, or whether the different dosage of picotamide (900 mg vs. 1200 mg in ADEP and DAVID, respectively) had a different impact on clinical outcome. Further studies are therefore necessary to explore the relationship between picotamide dosage and TxA_2 inhibition in vivo. In conclusion, activation of platelet arachidonic plays an important role in the pathogenesis of cardiovascular events in PAD, but the clinical trials with picotamide should be considered inconclusive and warrant further investigation.

Non-aspirin Drugs and PAD Drugs Inhibiting Platelet ADP Receptors

Thienopyridine is a drug category that inhibits platelet aggregation by interfering with platelet ADP receptors, i.e., $P2Y_1$ and $P2Y_{12}$.

Ticlopidine was the first drug of this class investigated in patients with PAD with the aim of evaluating the benefit on claudication or the cardiovascular events that complicate the clinical course of PAD. As far as the claudication is concerned, a prospective Italian study performed in 151 patients with claudication demonstrated that ticlopidine (250 mg/bid) increased walking distance compared to placebo-treated patients (Balsano et al. 1989). In the same trial, ticlopidine-treated patients' ABI did not worsen compared to placebo-treated patients, indicating that such antiplatelet treatment may favorably influence atherosclerotic progression. As far as the cardiovascular events are concerned, a Swedish trial (Janzon et al. 1990) investigated if ticlopidine (250 mg/bid), compared to placebo, was able to reduce cerebro- and cardiovascular events in a population with claudication. A total of 687 patients were included in the trial and followed up for 5 years. There was no difference in the clinical end points between the two groups. However, the secondary end point of mortality was lower in the ticlopidine group, and in an on-treatment analysis, there were fewer ischemic events in the ticlopidine-treated group.

The effect of thienopyridine on cardiovascular events was later examined in the CAPRIE trial, which compared the clinical efficacy of clopidogrel (75 mg/day), which is a derivative of ticlopidine, with aspirin (325 mg/day) in a population at different risks of cardiovascular events (CAPRIE 1996). One third of this population was affected by PAD. During 3 years of follow-up, clopidogrel was marginally significantly superior to aspirin in reducing cardiovascular events in the entire population (however the overall difference was insufficient to convince regulatory authorities that clopidogrel was indeed superior to aspirin). A post hoc analysis of the CAPRIE trial performed in the PAD population showed a significant risk reduction of 24 % for cardiovascular events in the clopidogrel-treated group compared to the aspirin group suggesting that the inhibitors of ADP receptors may be particularly efficacious in PAD patients.

In 2007, a publication of a post hoc analysis of Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance (CHARISMA) trial (Bhatt et al. 2007) included 2838 patients who had symptomatic PAD. Patients with PAD had a median time from diagnosis to the primary end point [cardiovascular death (including hemorrhagic death), myocardial infarction, or stroke (from any cause)] of 23.6 months in mean. The overall rate of cardiovascular death, MI, or stroke in PAD cohort was 8.7 % in the placebo plus aspirin arm and 7.6 % in the clopidogrel plus aspirin arm (HR = 0.869; 95 % CI: 0.671–1.125). Nevertheless, although the risk reduction in the PAD subgroup appeared similar to that observed in patients with prior MI or prior stroke, it did not reach statistical significance.

Together, these data indicate the clinical usefulness of thienopyridines in PAD to prevent major cardiovascular events. Given the level of evidence from the CAPRIE study, the FDA approved the use of clopidogrel in symptomatic patients with an ABI < 0.85 or PAD patients with a prior history of limb revascularization.

Clopidogrel (75 mg/day) is now recommended as a safe and effective alternative antiplatelet therapy to aspirin to reduce the risk of MI, ischemic stroke, or vascular death in individuals with symptomatic atherosclerotic lower extremity PAD, including those with intermittent claudication or critical limb ischemia, prior lower extremity revascularization, or prior amputation for lower extremity ischemia (Alonso-Coello et al. 2012; Rooke et al. 2011).

Moreover, the use of clopidogrel monotherapy or the use of clopidogrel plus aspirin and in PAD should be further assessed by PAD-specific interventional trials.

Nevertheless, in post hoc analysis of PLatelet inhibition And patienT Outcomes (PLATO) trial, patients with PAD ($n = 1144$) treated with ticagrelor showed numerically lower death from vascular cause, MI, or stroke when compared to treated with clopidogrel ones, but these results did not reach statistical significance (Patel et al. 2015).

The EUCLID (Examining use of tiCagreLor In paD) trial was designed to evaluate the benefit of ticagrelor 90 mg twice daily compared with clopidogrel 75 mg daily in 13,885 patients with symptomatic PAD with a median follow-up of 30 months. The primary efficacy endpoint was a

composite of cardiovascular death, myocardial infarction, or ischemic stroke, and the primary safety endpoint was major bleeding. The primary efficacy endpoint had a hazard ratio of 1.02 [95% confidence interval (CI) 0.92–1.13; $p = 0.65$], and major bleeding had an HR of 1.10 (95% CI 0.84–1.43; $p = 0.49$). Thus, there was no benefit of ticagrelor over clopidogrel in preventing major cardiovascular events indicating that clopidogrel was an active comparator and both drugs had similar results. There were two major subgroups enrolled, and those who had a prior revascularization had a risk of acute limb ischemia of 2.5% compared with 0.6% in patients enrolled based on an abnormal ankle-brachial index. The adjusted hazard ratio was 4.23 for the risk of acute limb ischemia in patients with a prior history of revascularization indicating a substantially increased risk based on a prior limb intervention. Ticagrelor was not superior to clopidogrel in preventing acute limb ischemia (Jones et al. 2016; Hiatt et al. 2016).

Non-aspirin Drugs and PAD Drug Antagonist of Protease-Activated Receptor-1

Vorapaxar is an oral, protease-activated receptor [PAR]-1 antagonist that inhibits thrombin-induced platelet aggregation. It has been recently shown to lower the occurrence of acute limb ischemia and peripheral revascularization in a subset of patients with PAD included in the Thrombin Receptor Antagonist in Secondary Prevention of Atherothrombotic Ischemic Events (TRA2°P)-TIMI 50 study. This is a randomized, double-blind, placebo-controlled trial of vorapaxar in 24,449 patients with stable atherosclerosis (Bonaca et al. 2013). A subgroup analysis of the study showed that in PAD patients ($n = 3787$), vorapaxar did not significantly reduce the risk of cardiovascular death, MI, or stroke, while the occurrence of acute leg ischemia was significantly reduced; vorapaxar therapy was, however, associated with increased risk of bleeding. These findings highlight a potential therapeutic approach to reduce acute limb ischemia and the need for peripheral revascularization in patients with symptomatic PAD. Based on the results of the trial, in May 2014, the US Food and Drug Administration (FDA) approved vorapaxar for the reduction of thrombotic cardiovascular events in patients with PAD.

Comparison of Efficacy of Antiplatelet Treatments in PAD

Although patients with PAD warrant lifelong antiplatelet therapy, an important issue relates to the potentially different impact of antiplatelet drug categories in the clinical progression of PAD. To investigate if specific antiplatelet treatment had a different impact on clinical outcomes, a meta-analysis has been performed in patients with claudication and/or ABI ≤ 0.99 (Basili et al. 2010). Twenty-nine clinical randomized trials on antiplatelet therapy for prevention of vascular death, myocardial infarction, and stroke in 10,735 peripheral artery disease patients have been included in the analysis.

The authors found 1900 (17.70 %) patients in trials with aspirin, 5326 (49.61 %) in those with thienopyridines, 2324 (21.65 %) in those with picotamide, and 1185 (11.04 %) in those with other antiplatelet drugs (including three studies where the active drugs were aspirin and/or dipyridamole). The results of this meta-analysis showed that in patients with claudication, antiplatelet treatment is efficacious in reducing vascular outcomes with a risk reduction of 17 %. Of note, analyzing separately each drug category, conclusive results were achieved by trials with thienopyridines that reduced the risk of cardiovascular events by 22 % ($p = 0.014$); a trend to a reduction was observed with aspirin (−15 %, $p = 0.208$) or picotamide (−21 %, $p = 0.302$), but these changes were not statistically significant. One of the limitations of this meta-analysis included the fact that it has been done in patients with stable PAD and could not, therefore, be extrapolated to nonmedical PAD series. Another limitation of the study was the lack of a direct comparison between two antiplatelet treatments that should require more interventional trials.

Conclusions

Antiplatelet therapy is indicated in patients with PAD to reduce the risk of major cardiovascular events. In patients with PAD who have clinical evidence of concomitant coronary or cerebrovascular disease, aspirin would be first-line.

It remains an open issue if PAD represents an atherosclerotic clinical model where aspirin, differently from coronary heart disease, is less effective in reducing atherosclerotic progression. Based on the reported results, further trials with aspirin should be done in symptomatic PAD patients. In these same patients, clopidogrel would be an acceptable alternative. However, the data supporting the use of antiplatelet drugs in patients with PAD, who do not have a history of other cardiovascular disorders, are still inconclusive, and further study is necessary to explore this issue.

Antiplatelet treatment should be indicated in PAD with clinically evident cardiovascular complication; aspirin or clopidogrel are the first-choice line for antiplatelet treatment. Conversely, the efficacy of antiplatelet treatment for primary prevention in symptomatic or asymptomatic PAD patients is less evident, and, thus, future interventional trials should analyze its cost-effectiveness in this clinical setting.

Take-Home Messages

Aspirin appears to have marginal benefits for reducing initial cardiovascular events when used for PAD patients without clinically evident coronary artery disease.

Clopidogrel significantly reduced cardiovascular events in the setting of peripheral arterial disease and would be an acceptable alternative to aspirin.

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Platelet Transfusions

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Abstract

Platelets clearly have an important role in hemostasis as evident by the increased risk of bleeding noted in severely thrombocytopenic individuals and in those with acquired or inherited defects in platelet function. Therefore, it was reasoned that platelet transfusions to such individuals may alleviate or prevent life-threatening bleeds. This chapter will review the history, the present, and the future of such transfusions as we transition from obtaining platelets from individual donors to growing custom platelets in culture. The opportunities that can arise from this new approach go beyond simply providing functional platelets tailored to meet individual needs and designed to avoid some of the pitfalls of current platelet transfusions and open up a new vista of potential therapeutic products targeted to sites of vascular injury.

History of Platelet Transfusion

Richard Lower performed the first successful blood transfusion in 1665 and kept a dog alive, by infusing blood from one dog into another (Spence and Erhard 2013). Just 2 years later, several people reported transfusing sheep blood into humans, usually with poor outcome and not always for the most ethical reasons so that the medical community initially banned experimentation with transfusion therapy (Spence and Erhard 2013). In 1818, James Blundell completed the first successful human blood transfusion in a series of eight women to manage postpartum hemorrhage (Blundell 1818), and in 1910 transfusions were recognized as successful therapeutic hemostatic interventions when Duke noted that they reduced the bleeding time (Duke 1910). It was not until 1962 that platelet transfusion began to be used more routinely, especially in cancer patients when the relationship between thrombocytopenia and hemorrhage was noted (Gaydos et al. 1962). In the 1970s, the combination of several observational studies identifying a possible role for prophylactic platelet transfusion in hypoproliferative thrombocytopenia and the discovery (Roy et al. 1973; Higby et al. 1974) that platelets were best stored at room temperature with gentle agitation to preserve function (Murphy and Gardner 1969) allowed for the proliferation of platelet transfusions as part of standard management of patients receiving chemotherapy (Blajchman et al. 2008). Further developments included reduction of the risks of bacterial contamination in units and developing strategies to limit platelet sensitization that markedly improved the efficacy of platelet products in individuals.

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Indications for Platelet Transfusions

This development of platelet transfusion therapy was driven, in large part, by the observation that hemorrhage due to thrombocytopenia was a leading cause of death in adults and children receiving chemotherapy (Gaydos et al. 1962; Freireich 2011). Today, platelet transfusions have become a routine part of practice in some settings, with the most common indication being prophylactic platelet transfusion in the setting of myelosuppressive chemotherapy. In some studies, 75–80 % of platelet transfusions were performed for this indication (Greeno et al. 2007; Whitaker 2013; Charlton et al. 2014). Critical illness, surgery, and massive trauma are other common indications that result in platelet transfusion. Therefore, platelet needs fall into two broad categories: prophylaxis to prevent bleeding in high-risk settings and interventional where bleeding is ongoing. Thus, one can envision that the ideal product for these two categories might be distinct.

Despite the fact that transfusions have been in use for many years, there is still considerable debate about ideal platelet transfusion triggers, doses, and even the appropriate use of prophylactic versus therapeutic transfusions. The AABB (formerly American Association of Blood Banks) recommends a prophylactic platelet transfusion trigger of $<$10,000/μL in adult inpatients with chemotherapy-induced hypoproliferative thrombocytopenia, with higher thresholds of 20–50,000/μL for patients undergoing procedures (Kaufman et al. 2015). Other sources recommend a platelet transfusion trigger of $<$10,000/μL for stable, non-bleeding patients and $<$20,000/μL for febrile patients (Qureshi et al. 2007; Nahirniak et al. 2015). Higher thresholds have been recommended for neurosurgical patients (Qureshi et al. 2007).

Thrombocytopenia has been associated with increased mortality in critically ill patients (Thiolliere et al. 2013). Most interestingly, the platelet metric associated with poor prognosis in intensive care units is a 30 % decrease in platelet count (irrespective of starting platelet count or development of absolute thrombocytopenia) (Strauss et al. 2002; Moreau et al. 2007). However, in contrast, risk of bleeding is poorly correlated with degree of thrombocytopenia in this patient population (Strauss et al. 2002; Stanworth et al. 2013), and prophylactic platelet transfusions may not significantly alter the risk of bleeding, but may increase thrombotic complications and other problems (Cook et al. 2005). Part of the problem resulting in these seemingly contradictory findings may be the different settings being analyzed, the heterogeneity of platelet efficacy in different individuals, and the differences in platelet biology in various units based not only on the donor, but on the difficulty of maintaining highly reactive platelets ex vivo (Devine and Serrano 2010). Platelet transfusion in the setting of acute, nontraumatic illness is also complicated by increased platelet consumption/turnover, and in this setting, platelet transfusion in a non-bleeding patient may not be effective or necessary (Thiele et al. 2013). Recommended thresholds are not only important to balance the risks of bleeding versus the risks of thrombosis, exposure to potential contaminants and platelet sensitization, but also because of the recognition that provision of donor-based platelet transfusions may be limited in many western world countries that have aging populations with increasing needs for transfusion and a shrinking donor pool (Amrein et al. 2012).

Platelet Preparation

Advances in collection techniques, storage techniques, and additives to decrease platelet reactivity and bacterial contamination have made platelets safer. Today, more than four million units of platelets are transfused per year worldwide (Sullivan et al. 2007). Platelets for transfusion can be derived from whole blood donations or by single-donor apheresis with particular advantages to each technique. Whole blood-derived platelet units are obtained by pooling platelet-rich plasma or buffy coats from multiple donors using differential centrifugation steps. The platelets are then resuspended in plasma or an additive solution plus plasma. These units are relatively cheap to produce as well as optimize use of the entire unit of donated whole blood. However these units do not allow for HLA/HPA matching and result in increased donor exposures to foreign antigens and risk of platelet sensitization (Pietersz 2009). With single-donor apheresis units, the entire unit of platelets is obtained by apheresis from a single donor who needs to undergo a longer and somewhat more invasive procedure (Pietersz 2009). The production of these units is more expensive, and its hemostatic efficacy is dependent on that individual donor, but this method allows for HLA/HPA matching and minimizes the number of donors to which a recipient is exposed. Platelet count specifications for what constitutes an acceptable unit of platelets vary somewhat from country to country, but generally are $2–3 \times 10^{11}$ platelets/unit (e.g., in the United States, minimum platelet specifications are for 3×10^{11}/unit platelets, while Switzerland requires $>2.4 \times 10^{11}$/unit).

Platelet Storage and Its Effects on Platelet Function

In contrast to all other blood components, platelets are stored at room temperature with gentle agitation. They have a short shelf life of 5–7 days due to the risk of bacterial contamination (Perez et al. 2001). Unfortunately, while storage of platelets at room temperature appears to preserve their function, there is still damage to the platelets as a result of their

Table 1 Platelet storage lesion

Factors responsible for the platelet storage lesion	
Intrinsic	Calcium shift
	Signaling (centrifugation)
	Loss of platelet granules
	Cytoskeletal changes
Extrinsic	Metalloproteinase cleavage of receptors
	Hypoxic injury
	Platelet apoptosis

sensitivity to manipulation. This platelet storage lesion is poorly defined and likely multifactorial. Platelet centrifugation during the preparation process results in some degree of platelet activation causing calcium shifts and signaling (Hoffmeister et al. 2003). Also potentially contributing to the platelet storage lesion are metalloproteinase cleavage of the extracellular glycocalicin domain of glycoprotein Ib alpha (Sandgren et al. 2007), degradation of the cytoskeleton (Handigund and Cho 2015), platelet apoptosis (van der Wal et al. 2010), lactic acid accumulation and platelet hypoxia (which are partially ameliorated by agitation of the bag) (Hossien et al. 2014), and platelet degranulation (Handigund and Cho 2015) (Table 1).

Factors Affecting Transfusion Efficacy

Platelet efficacy can be assessed as either the degree of platelet count rise or the effect on bleeding. While assessing bleeding is arguably the most relevant, measuring platelet increment is the easiest in the vast majority of patients who are receiving transfusions because of thrombocytopenia. Intuitively, physicians feel that giving more platelets at a single time should increase the platelet increment and be more effective at preventing bleeding. This was demonstrated not to be true. According to the dose of prophylactic platelet transfusion (PLADO) trial (Slichter et al. 2010), increasing platelet dose from 1×10^{11} platelets/dose to 4×10^{11} platelets/dose did not alter the incidence of bleeding in patients getting prophylactic platelet transfusion, but resulted in more units of platelets being transfused in those patients getting higher platelet doses. A subanalysis showed that this was true even in pediatric patients, but that there was increased bleeding in children in general compared to adults (Josephson et al. 2012). This suggests that giving more platelets at a time in the setting of thrombocytopenia for prophylaxis is not helpful. Most guidelines recommend that platelets transfusions be matched for ABO blood group whenever possible because this increases the platelet recovery after transfusion (Julmy et al. 2009). Other factors that have been shown to decrease platelet increment after transfusion include irradiation of platelets, addition of certain additives, pathogen reduction, and length of time in storage, although most of these factors have not been definitely shown to impact platelet transfusion effectiveness in clinical bleeding (Triulzi et al. 2012). Finally, several patient factors, including gender, height/weight, as well as some clinical conditions and drugs, can also influence the platelet increment posttransfusion (Stanworth et al. 2015).

The greatest cause of platelet transfusion refractoriness is platelet sensitization. Refractoriness is a failure to achieve the expected platelet increment from transfusion (generally for at least two consecutive platelet transfusions of ABO-matched platelets). There are several nonimmune and immune factors that can contribute to platelet refractoriness and should be considered in the assessment of patients. Generally, the nonimmune factors that contribute to platelet refractoriness cause platelet consumption and include bleeding, infection/sepsis, splenomegaly, and graft-versus-host disease (Doughty et al. 1994). Drugs may also contribute to these mechanisms in particular, vancomycin, heparin, and the $\alpha_{IIb}\beta_3$ inhibitors (Stanworth et al. 2015). In approximately 20 % of patients, immune mechanisms play a role, most commonly due to HLA antibodies (Hod and Schwartz 2008). Risk factors for immune refractoriness include a history of prior transfusion, pregnancy, and/or transplantation (Hod and Schwartz 2008).

Platelet Transfusion Risks/Reactions

There are good reasons, beyond conservation of a limited and precious resource, decreasing healthcare costs, and optimizing utilization, to limit the number of transfusions. While platelet transfusions are generally safe, they are not completely without risk. Platelet transfusions account for 10 % of transfusions, but are responsible for 25 % of transfusion-associated adverse events (Harvey et al. 2015).

Febrile nonhemolytic transfusion reactions (FNHTR) to platelet concentrates commonly occur and have been attributed to the presence of cytokines in the plasma from residual leukocytes. These adverse reactions increase in frequency and severity with length of storage (Kelley et al. 2000). The concentration of the cytokines interleukin-6 (IL-6), tissue necrosis factor alpha (TNFα), and interleukin-1 beta (IL-1β) increase with storage, particularly in platelet concentrates containing $>3 \times 10^9$/L leukocytes (Kunz et al. 1998; Kanter et al. 2008). Cytokine accumulation is most marked after day 3 of storage, and levels are highest at day 5 (Kanter et al. 2008). Leukocyte depletion of platelet products using product filtration has been shown to reduce the incidence of FNHTR (Mishima et al. 2015); in the presence of adequate leukocyte depletion of platelet products ($<5 \times 10^6$/unit), the incidence of FNHTR is not affected by platelet storage time. The type of platelet product

(random donor versus apheresis platelet product) has no effect on the incidence of FNHTR.

Platelet products can also potentially be contaminated with infectious agents. Bacterial contamination occurs in ~1 in 2000 platelet units. Despite active and passive surveillance systems to detect bacterial contamination in platelet units, septic transfusion reactions still occur in ~1:50,000 platelet transfusions (Klein 2005) and are associated with a fatality rate of 1.3 deaths per million platelet transfusions (Hong et al. 2016). Viruses are also carried by contaminating leukocytes as in whole blood. Although the risks of viral transmission are fairly low, there are always concerns about transmission of the next new viral infection (Klein 2005). The current discussions of variant Creutzfeldt-Jakob disease suggest that the potential infective agent is carried by platelets (Jones et al. 2005). Finally, as with all blood products, allergic reactions, due to IgE and IgG antibodies in the recipient against plasma proteins in the transfused blood component, or due to transfusion of cytokines, chemokines, and histamine generated in the platelet product during preparation and storage, occur in 0.09–21 % of transfusions and appear to be donor unit and patient specific and should be treated similarly to allergic reactions with any other blood product (Domen and Hoeltge 2003).

Transfusion-associated acute lung injury (TRALI) is the leading cause of transfusion-related mortality in the United States that can occur following a platelet transfusion and has been reported with an incidence of 2.8 per 100,000 apheresis platelets where universal donor restriction policies have not yet been fully adopted (Muller et al. 2015). TRALI involves the appearance of acute respiratory distress, new bilateral pulmonary infiltrates in the absence of circulatory overload within 6 h of transfusion, and can occur with transfusion of any blood component. The rate of TRALI is highest in platelet and plasma transfusions (Stolla et al. 2015). This is generally associated with anti-leukocyte antibodies in the donor, which are most frequently found in donors who have a history of pregnancy, but there may be antibody negative TRALI in about 20 % of cases (Peters et al. 2015a, b). The current leading hypothesis on pathophysiology for TRALI puts forth a "two-hit" or "threshold" model of pathogenesis where the transfusion recipient sustains the "first hit" or reaches a "threshold" of susceptibility to TRALI through critical illness, sepsis, or cardiac surgery, for example, and then receives a transfusion with a product that has requisite characteristics (the "second hit"). These two models are illustrated in Fig. 1 (Bux and Sachs 2007). The screening of at-risk donors has resulted in some countries in dramatic decreases in the frequency of TRALI (Muller et al. 2015); however, there are still cases of nonimmune TRALI and even immune TRALI that are not prevented by screening programs. The risks of platelet transfusions are summarized in Table 2.

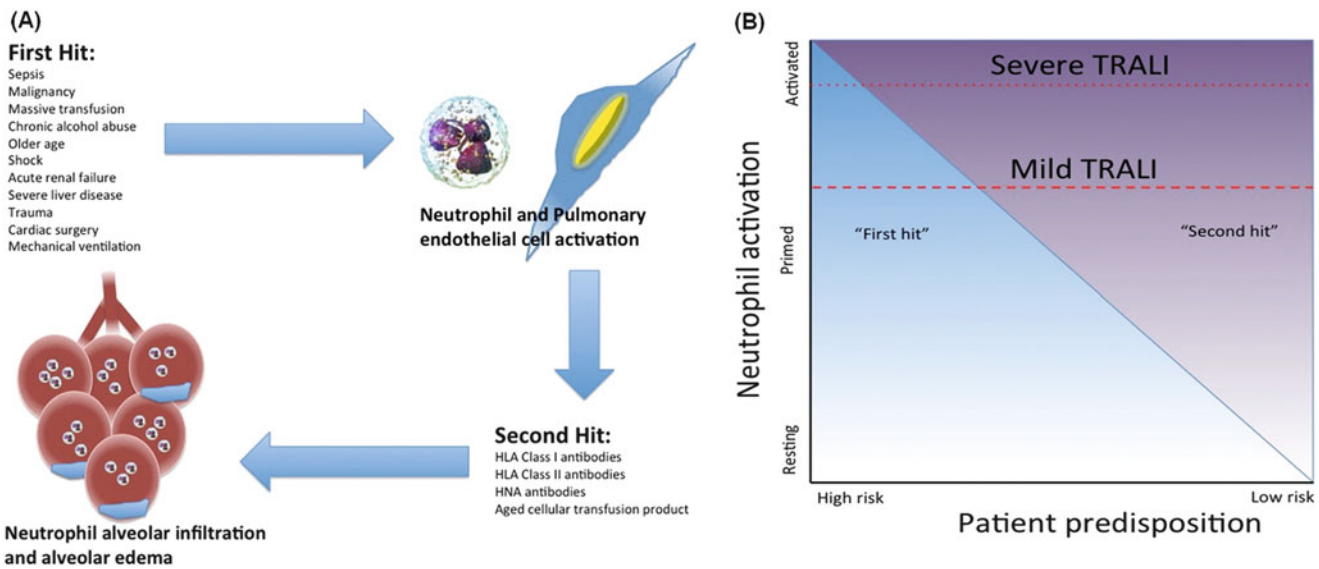

Fig. 1 (**a**) The two-hit model of transfusion-related acute lung injury (TRALI) with hypothesized pathophysiology. A "first hit," an underlying clinical condition of the patient, results in priming of neutrophils and activation of pulmonary endothelial cells. The "second hit," the transfusion of a blood product, causes activation of the neutrophils and coagulation pathways resulting in TRALI. Neutrophil activation results in migration of neutrophils through the interstitium into the alveoli, which are filled with protein-rich edema. The "second hit," the transfusion product, may contain accumulated cytokines, aged cells, human leukocyte antigen (HLA) antibodies or human neutrophil antigen (HNA) antibodies. (**b**) The threshold model of TRALI. In the "threshold model" the threshold is formed by the level of priming of lung neutrophils and the ability of the mediators in the transfusion product to activate these primed neutrophils

Table 2 Risks of platelet transfusion

Risks of platelet transfusions		
Transfusion reaction	Frequency	Postulated mechanism
Febrile nonhemolytic transfusion reaction	1.1–4.6 % of transfusions	Accumulation of cytokines
Allergic reaction	0.09–21 % of transfusions	IgE/EgG antibodies in recipient against plasma proteins in platelet product; transfusion of cytokines, chemokines, and histamine in product during preparation and storage
TRALI	2.8 per 100,000 apheresis units	Multifactorial but includes transfusion of HLA or HNA antibodies
Bacterial contamination	~0.5 %; 1:50,000 transfusions	Due to storage at room temperature

In Vitro Platelet Production

Clearly, donor-based platelet transfusions have made an important contribution to patient care, but have also been limited by the quality and safety of the final product and by the increasing demand for platelets by an aging clinical population and a relatively fixed platelet donor-pool size. One direction to overcome these limitations and to offer personalized platelets has been to produce platelets in vitro from hematopoietic or other stem cells. While there has been important progress made in the area of in vitro platelet production, the technology and methods to produce sufficient quantities of functional platelets in culture have yet to be fully realized. Below, we describe the current status of in vitro platelet production and the remaining challenges that still need to be surmounted to making sufficient quantities of functional platelets in culture. We will also discuss additional application that can come from this alternative source of platelets.

CD34+-Derived Megakaryocytes and Platelets

Thrombopoietin (TPO) was first proposed to be an important regulator of platelet production in 1958, but was not isolated and characterized until 1994, confirming that it is an important regulator of megakaryopoiesis and thrombopoiesis (Kaushansky and Drachman 2002). Recombinant TPO began to be used in culture to produce and expand megakaryocytes and platelets beginning with CD34+ cells isolated from different sources, including mobilized peripheral blood, adult bone marrow, and umbilical cord blood. These sources showed variable yields of large megakaryocytes with polyploid nuclei and also yields of in vitro-released particles that we will term "platelet-like particles" or PLPs (Su et al. 2001; De Bruyn et al. 2005). Choi et al. first showed that these peripheral blood CD34+-derived megakaryocytes in the presence of TPO could produce PLPs after 12 days of culture (Choi et al. 1995). While the size range of these particles was not shown, Choi showed that thrombin or ADP could not only activate a portion of the PLPs but also participate in $\alpha_{IIb}\beta_3$-mediated aggregation. Comparative studies were done with donor-derived platelets, but with at least a portion of the platelets being pre-activated. An additional problem was that it was unclear the amount of PLPs being released per initial CD34+ cell or per derived megakaryocyte and whether these PLPs could potentially be used for transfusions. In 2006, Matsunaga et al. showed that large numbers of PLPs could be produced from cultured umbilical cord blood-derived CD34+ cells (Matsunaga et al. 2006). In their 33-day, three-step protocol, ~3–4 $\times$ 10^4 PLPs could be generated from one umbilical cord CD34+ cell after culture on stromal cells but only a portion were CD41+. In this study, they also showed functionality of these platelets through ADP activation by surface p-selectin binding and proper CD41 integrin function through the monoclonal antibody PAC-1 binding to the activated cells. Experiments were done that compared in vitro-generated PLPs to donor-derived platelets and showed that in vitro PLP production for possible clinical use from CD34+ cells is feasible, but neither paper showed in vivo studies.

Embryonic Stem, Induced Pluripotent Stem (ES/iPS) Cells, and Other Stem Cells Differentiate into Megakaryocytes

Human embryonic stem cells were generated from fetal material, and iPS cells were first derived by Yamanaka et al., from somatic cells in 2006 by transiently expressing four factors in these cells (Takahashi and Yamanaka 2006). ES/iPS cells are appealing in that they can replicate for many generations and can also be differentiated into a variety of different lineages, including the hematopoietic lineage (Holden and Vogel 2008; Osafune et al. 2008; Lim et al. 2013). A number of groups have reported making megakaryocytes from ES/iPS cells using protocols incorporating TPO in the final differentiation process, releasing PLPs (Takayama and Eto 2012; Nakamura et al. 2014). Unlike CD34+ cells that have a limited in vitro life span, ES/iPS cells can theoretically be greatly expanded

prior to differentiation into megakaryocytes, and it was hoped that these stem cells would be a useful resource to obtain clinically relevant numbers of platelets (Nakamura et al. 2014). Initial studies with iPS cell-derived megakaryocytes have shown several limitations: only 1–10 hematopoietic progenitor cells can be produced per initial ES/iPS cell (Lu et al. 2011; Takayama and Eto 2012; Wang et al. 2015), and only 1–10 megakaryocytes can be produced from initial hematopoietic progenitor cell (Lu et al. 2011; Amabile et al. 2013). Finally, these megakaryocytes are small and of low ploidy (Wang et al. 2015), and only 1–10 PLPs could be generated per megakaryocyte. In vitro studies generally focused on individual PLPs to show similar size, shape, and organization of particles to donor-derived platelets and to show functionality. When the full population was examined, only a few percent of the cells were CD41+ and even a smaller percent were CD41+CD42+ (Lev et al. 2011; Lu et al. 2011). Moreover, these PLPs had minimal reactivity to various agonists relative to what one would expect of donor-derived platelets (Gaur et al. 2006). Finally, unlike infused donor-derived platelets, human PLPs infused into immune-compromised NSG mice had a markedly shortened half-life consistent with the fact that most of the CD41+CD42+ PLPs were actually CD42b-, likely due to metalloproteinase cleavage in culture (Fuentes et al. 2010). In vivo functional studies did indicate active incorporation of these PLPs into thrombi in mice (Mishima et al. 2015), but the efficacy of this process relative to donor-derived platelets was not tested.

Other sources of stem cells have also been used in place of ES/iPS cells. These have successfully included endometrial stromal stem cells, fibroblasts, and adipocytes (Mali et al. 2008; Matsubara et al. 2009; Wang et al. 2012). These cells have yielded similar results as ES/iPS cells in terms of efficacy of amplification of megakaryocyte yield per initial stem cell and yield of PLPs per megakaryocyte. The biology of these PLPs in culture and in vivo was also similar to those seen with ES/iPS cell-derived PLPs.

Overcoming the Limitations of In Vitro Megakaryopoiesis and Thrombopoiesis

One clear limitation with ES/iPS cell-derived hematopoietic lineages is that they replicate embryonic hematopoiesis (Sturgeon et al. 2014; Lachmann et al. 2015). There are megakaryocytes in the embryo (Tober et al. 2007), but whether they yield platelets with similar functionally to adult platelets is not clear. Technologies to derive definitive hematopoietic lineages have been described (Lachmann et al. 2015), but whether generating megakaryocytes from these cells would affect yield and/or quality of the final PLPs is unknown.

Another limitation is the yield of PLPs per beginning iPS cell is extremely low. Perhaps if one can establish a self-replicating intermediate cell from iPS cells that can proliferate for multiple rounds before differentiating into megakaryocytes, one can overcome this limitation. In 2014, Nakamura et al. showed that iPS cell-derived megakaryocyte progenitors expressing the proto-oncogene MYC under a doxycycline-inducible promoter and BMI1, a repressor of c-Myc-regulated apoptosis factors, could result in an expandable intermediate lineage (Nakamura et al. 2014). Upon removal of doxycycline, these cells differentiated into megakaryocytes and released PLPs. However, these PLPs had the same limitations as described for PLPs released without this intermediate expansion from iPS cells (Lu et al. 2011). In another approach to accomplish a similar expansion of an intermediate lineage strategy, Noh et al. showed that mouse ES cells expressing *Gata1* shRNA transgene under a doxycycline-inducible promoter could also undergo differential arrest at the megakaryocyte-erythroid progenitor stage, and, following terminal differentiation, these cells could become polyploid megakaryocytes that yielded platelets that were functional both in vitro and in vivo upon infusion (Noh et al. 2015). Whether these studies could be recapitulated in human cells and would yield actual platelets remains unknown.

Bioreactor Systems to Generate Platelets

Several groups have developed bioreactor systems to generate platelets from in vitro-grown megakaryocytes (Nakagawa et al. 2013; Thon et al. 2014; Di Buduo et al. 2015). These systems physically push megakaryocytes using flow through an uncoated plastic sieve in the presence of shear stress and collect the resulting particles as well as whole megakaryocytes at the efferent end. These preparations do contain some PLPs of the size of donor-derived platelets and have some in vitro responsiveness to agonist as well as adherence and spread on fibrinogen-coated surfaces comparable to growth in static cultures. However, the comparative size distribution and range, as well as the relative functionality compared to donor-derived platelets and in vivo half-life and functionality in recipient immunodeficient mice are missing from these reports. Another approach has used silk strands as opposed to a plastic sieve has been pursued and has also met with partial success (Pallotta et al. 2011; Di Buduo et al. 2015).

Infusing Megakaryocytes Rather than Platelets

Most groups assume thrombopoiesis occurs at the endothelial lining of the bone marrow (Machlus and Italiano 2013). The endothelial cells in the marrow sinuses have pores in them that may allow migration of cells in and out of the

intramedullar space. Intravital microscopy of megakaryocytes at these junctions has supported the migration of megakaryocytes across the endothelial cell layer and the immediate release of platelets or larger cytoplasmic fragments (Tavassoli and Aoki 1989; Junt et al. 2007); however, these studies are open to interpretation and can be used to also support the concept that whole megakaryocytes may migrate out of the marrow space intact or in large fragments and lodge in the pulmonary microcirculation and release platelets there (Zucker-Franklin and Philipp 2000). Fuentes et al. documented that both mouse- and human-infused megakaryocytes actually lodge in the lungs and release platelets there over the next few hours (Fuentes et al. 2010). It was also found that unlike in vitro-released PLPs, released human platelets are very similar in half-life, size, size distribution, and functionality in the recipient mice to donor-derived platelets. The mice tolerate these infusions well as only <5 % of the pulmonary vasculature need be occluded. Further larger animal studies will better define the clinical applicability of directly infusing megakaryocytes rather than depending on platelets to be produced ex vivo. Since peak platelet release may take up to 4 h, this approach may be more conducive for prophylactic therapy than for acute bleeding emergencies.

Other Uses of In Vitro-Generated Platelets

Platelet infusion is mostly to treat individuals with thrombocytopenia and rarely to treat patients with inherited defects in platelet function. However, using ES/iPS cells or other stem cells can now be modified by gene editing technologies to knock out or down a particular protein or to express a native or ectopic protein. These modified platelets may have biological and clinical uses that cannot be duplicated by donor-derived platelets.

Decreased Risk of Platelet Sensitization One of the complications of multi-transfusion of platelets is the development of antibodies to HLA markers on the cell and subsequent refractoriness to platelet transfusion. The elimination of the beta2-microglobulin gene in stem cells and their subsequent megakaryocytes and PLPs, after differentiation, has been reported (Riolobos et al. 2013; Feng et al. 2014). If these platelets have normal biology and functionality, they may become another reason to replace donor-derived platelets with a product less likely to lead to sensitization, especially in individuals likely to require multiple platelet transfusions.

Prolonging the Half-Life of Infused Platelets It is possible that overexpression of an antiapoptotic factor such as Bclx (Nakamura et al. 2014) or of the "do-not-eat-me" surface marker CD47 (Olsson et al. 2005) may improve the life span of platelets and reduce the frequency with which individuals need to receive transfusion.

Targeted Delivery of an Ectopic Protein in Platelets Several proteins have been ectopically expressed in megakaryocytes and platelets to achieve targeted delivery vehicle. Urokinase (Kufrin et al. 2003) and ADAMTS13 (Laje et al. 2009) have both been ectopically expressed during megakaryopoiesis, and both are stored in platelet alpha granules. Both can have a targeted and potent antithrombotic effect without causing systemic fibrinolysis for urokinase or von Willebrand factor polymer degradation for ADAMTS13. Ectopic expression of factor VIII (FVIII) has also been achieved in platelets, and it is stored in alpha granules (Yarovoi et al. 2003). This platelet FVIII is not released unless the platelet is activated and has been shown in mice models to significantly correct the bleeding diathesis in hemophilia A mice even in the presence of significant levels of circulating FVIII inhibitors (Shi et al. 2006; Gewirtz et al. 2008). Finally, platelets are known to enhance tumor cell metastasis (Zaslavsky et al. 2010). Platelet transfusions to thrombocytopenic patients with tumors may theoretically enhance hematogenous tumor spread (Lyman and Khorana 2009; Zaslavsky et al. 2010). The ectopic expression and release of an anti-angiogenic agent like a soluble VEGFR may prevent that complication, and it may be useful to incorporate such a modification into platelets being given to cancer patients undergoing chemo- or radiotherapy.

Future Perspective

The clinical need for platelet products continues to expand as our population continues to age and as new therapeutic options are developed that induce thrombocytopenia. The size of the platelet donor pool is likely to shrink concurrently. While alternative therapies that can ameliorate bleeding risk in these patients may develop utilizing TPO mimetic drugs to enhance production (Kuter 2009) or blocking the pathways for platelet clearance (Grozovsky et al. 2015), another potential approach is to generate platelets in cultured megakaryocytes derived from a variety of stem cell sources perhaps ES/iPS cells. Such substitute platelets may overcome other issues related to donor-derived products including high-quality product standardization and ability to avoid sensitization. They also offer the opportunity to develop personalized platelets and targeted drug delivery via platelets to expand the uses of platelet transfusion. It would be anticipated that the advantages of these products would compensate for their likely greater costs of production compared to platelets from volunteer donors.

Technologies to develop a self-proliferating, intermediate cell line in vitro that can give rise to functional megakaryocytes and platelets appear to be an important tool that would allow the scale-up needed to provide a sufficient number of platelets for clinical needs. Limiting or eliminating injury to the megakaryocytes in culture and subsequently to the derived platelets is also important. A way to release functional platelets from these megakaryocytes in vitro or to explore further the clinical applicability of direct megakaryocyte infusions must also be developed. Importantly, a comparative gold standard platelet product that is quiescent prior to being exposed to agonists and is highly responsive to agonists is required to judge any in vitro-generated product. An acceptable in vitro-derived product must be at least comparable to this standard platelet preparation, not only in agonist responsiveness but also in size range and distribution, in the absence of contaminating non-platelet cell fragments, and in circulating half-life. At the moment, none of the in vitro products would meet such criteria or were not tested against such high standards, but with further growth in our understanding of stem cell differentiation to adult hematopoietic progenitor cells, and of the underlying biology of megakaryopoiesis and thrombopoiesis, it would be reasonable to expect that such a clinically applicable product would emerge.

Take-Home Messages

- More than four million platelet units are transfused worldwide on a yearly basis.
- Advances in platelet collection, storage, and additives to decrease platelet activation and bacterial contamination have made platelet products safer.
- Febrile nonhemolytic transfusion reactions are likely reduced by leukodepletion but not by use of single-donor versus random-donor platelet units.
- TRALI has complex pathophysiology and is dependent upon both recipient and donor factors.
- Research to develop other sources of platelets for transfusion is ongoing and may lead to better platelet products in the future.

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Platelet-Rich Plasma in Regenerative Medicine

Joseph Alsousou and Paul Harrison

Abstract

The clinical application of platelet-rich plasma (PRP) has been increasing sharply in the last two decades. Its role as a potential regenerative agent and ease of application have allowed it to take huge share in the fast-evolving biological therapy field. The reported effect of PRP on a range of tissue types including bone, cartilage, tendon and muscle has attracted clinical interest in fields such as trauma, orthopaedic, maxillofacial and plastic surgery where effective healing of tissues is critical for successful outcome. The results of in vitro and animal studies that largely report positive effects of PRP on cellular and matrix regeneration have been the main drive for its translation to clinical settings. Despite the lack of appropriately powered trials, PRP administration remains an attractive strategy given its cost-effective, minimally invasive nature and the autologous nature of PRP. In this chapter, the current literature on the use of PRP in regenerative medicine is reviewed highlighting both some of the controversy surrounding this approach and some emerging clinical applications. A new PRP classification system is presented to allow better description of the variable clinical PRP products and their correlated outcome.

Introduction

Platelets are increasingly recognised to play important roles in physiology beyond promotion of haemostasis and thrombosis. Platelet-rich plasma (PRP), an autologous derivative of whole blood that contains a supraphysiological concentration of platelets, has gained increasing attention in both the scientific literature and the wider media for its potential application as a regenerative adjunct therapy (Alsousou et al. 2009; Engebretsen et al. 2010). The regenerative effect of PRP exerted by producing a local environment for tissue regeneration which is rich in growth factors and other cytokines has been supported by in vitro and animal studies that suggest a positive influence on the migration and proliferation of a number of cell types. Furthermore, the full array of potential bioactive growth factors and chemokines released upon platelet activation is becoming well defined (Coppinger et al. 2004; Nurden 2011). These include transforming growth factor (TGF-ß1 and TGF-ß2), platelet-derived growth factor (PDGF-AA, PDGF-AB and PDGF-BB), vascular endothelial growth factor (VEGFs A and C), insulin growth factor (IGF-1) and epidermal growth factor (EGF). These factors can promote local angiogenesis, stem cell homing and local cell migration, proliferation and differentiation, coupled with the deposition of matrix proteins such as collagen which all play a key role in enabling the restoration of normal tissue structure and function (Coppinger et al. 2004; Nurden 2011).

The reported clinical use of PRP is largely confined to the last two decades and initially centred on its application in dental and maxillofacial surgery (Gibble and Ness 1990; Sanchez et al. 2003). More recently, regenerative effects of

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PRP in a range of tissue types including bone, cartilage, tendon and muscle have attracted interest in fields such as orthopaedic and plastic surgery where effective restoration of sometimes poorly vascularised and damaged tissue is a critical determinant of successful clinical outcome (Alsousou et al. 2013; Gentile and Cervelli 2010). Despite the lack of appropriately powered trials, PRP administration remains an attractive strategy given its cost-effective, minimally invasive nature and the autologous nature of PRP. The latter eliminates concerns about the potential for disease transmission or immunological reactions. In this chapter, the current literature on the use of PRP in regenerative medicine is reviewed highlighting both some of the controversy surrounding this approach and some emerging clinical applications.

PRP Definition

Platelet-rich plasma (PRP), which is also known as platelet-enriched plasma (PeRP), platelet-rich concentrate (PRC), platelet concentrate, leucocyte-rich PRP (L-PRP), platelet-rich fibrin (PRF), platelet rich in growth factors (PRGF), platelet-rich fibrin matrix (PRFM), autologous concentrated plasma (ACP), platelet gel or platelet releasate, may be defined as a volume of the plasma fraction of autologous blood having a platelet concentration above baseline (Marx et al. 1998). A high concentration of (1,407,640 ± 320,100/μl) of platelets in plasma has been suggested to be a working definition of PRP (Weibrich et al. 2002). Although platelet-rich plasma refers to autologous preparations, the preparation of allogeneic PRP (aPRP) might not be excluded. The use of aPRP may serve as an alternative option in the case of patients who refuse to be subjected to a blood drawing procedure or have contraindications to autologous PRP (Dragica et al. 2007).

In clinical practice, there are a large number of PRP products obtained using different preparation methods. Although they are all labelled as PRP; the content, purity and hence the biological properties of those products all vary widely and could impact upon their potential efficacy. This is causing confusion among clinicians and scientists when it comes to reporting the clinical outcome of PRP applications. Several clinical trials have been conducted without clear quantification of the used PRP biological properties, which may differ significantly leading to varying outcomes. Table 1 shows a list of PRP preparation devices and the related product content. In this chapter, we will describe recently suggested classifications and then suggest a new system to classify PRP based on its biological components.

Cellular Components of PRP

PRP regenerative properties are based on the production and release of bioactive proteins including multiple growth and differentiation factors, following platelet activation. However, the role of other cells that may exist in PRP such as leucocytes and erythrocytes is still under investigation (Alsousou et al. 2010). The presence of leucocytes has a great impact on the biology of these products, not only because of their immune and antibacterial properties but also because they are key players in tissue healing and local factor regulation.

Most PRP products have varying concentrations of blood cells (platelets, leucocytes and erythrocytes) and cell-derived microvesicles, and many of them also have high concentrations of leucocytes. The direct cellular interaction between platelets, leucocytes and target tissues is still largely unknown (Bielecki et al. 2012). Polymorphonuclear leucocyte (PMN) and monocyte adhesion to activated platelets is important for the recruitment of PMN and monocytes at sites of tissue damage. The binding of activated platelets to PMN/monocytes involves P-selectin and the activated β_2-integrin CD11b/CD18 that not only results in transactivation but may cause significant changes in patterns of gene expression to trigger inflammation, e.g. tissue factor expression on monocytes. The effect of PRP components on cells and tissues is a complex process that involves growth factors and cytokines produced by both platelets and leucocytes. In addition, there may be direct cellular interactions that also regulate the intracellular signalling pathways in the resident tissue cells (e.g. ERK and AkT pathways for proliferation) (Franklin et al. 2012).

Bioactive Proteins and Growth Factors in PRP

Researchers have identified hundreds of potentially bioactive proteins either inside platelets or on the plasma membrane (Nurden 2011). The most commonly studied platelet proteins include PDGF, TGF-β, PDEGF, VEGF, IGF-1, FGF and EGF and some cytokines including proteins such as platelet factor 4 (PF4) and CD40L. Chemokines and newly synthesised metabolites are also released during platelet activation. PRP also facilitates the application of autologous plasma along with platelet-derived proteins. In addition, it promotes the development of a fibrin scaffold at the desired location that can act as a temporary matrix for cell growth and differentiation to assist repair in the injured tissue. Studying the role of those bioactive factors is essential to understand the biological activities of PRP.

Table 1 List of PRP preparation devices and their PRP products

Technology	Device name	Product name	Concentration increase[a]	Platelet recovery (%)	Product content
Floating buoy or shelf	Biomet GPS™	PCP	3.2×	70 %	Manual collection of buffy coat which contains platelets, WBC and minimal amount of RBC
	Harvest® SmartPRep2 BMAC™ Depuy Symphony II 3i PCCS	PRP	4.6× 4.0× 4.0×	65–72 %	
Cell saver-based systems	Electa, Haemonetics, CATS, BRAT	PRP	4–6×	75 %	Platelet concentrate only with plasma. No WBC or RBC contamination
Computer-aided system	Sorin Angel	PRP	4.3×	70 %	Automated buffy coat collection, which contains concentrated platelets, WBC fractions and minimal amount of RBC
	Arteriocyte Medical (Magellan™)	PRP	5.1×	76 %	
Standard centrifugation	AutoloGel System SmartPReP	PRP PReP	1–2×	78 %	Platelets in plasma suspension with minimum white cells and low concentration of platelets
	Cascade PRFM Fibrinet system	PRFM	1–2×	78 %	Platelet-rich fibrin membrane
	Choukroun's PRF	PRF	1–2×	70 %	Leucocyte- and platelet-rich fibrin
Direct siphoning	GenesisCS	PRP	6×	68 %	concentrate of platelets and leucocytes through siphoning device
Direct aspiration	Secquire Arthrex ACP	PRP	1.6×	31 %	Manual aspiration of platelet and plasma after centrifuging
Platelet separation	Vivostat	PRF FS	6×	65 %	Platelet-rich fibrin Fibrin sealant without platelet
Platelet filtration	Caption Advanced Tissue Regeneration (ART)-Curasan Set	PC	4.3×	–	Concentrated platelets without plasma

ACP autologous concentrated plasma, *PCP* platelet concentrated plasma, *PRF* platelet-rich fibrin, *PRFM* platelet-rich fibrin matrix, *PRGF* plasma rich in growth factors, *PRP* platelet-rich plasma, *RBC* red blood cells, *WBC* white blood cells

[a]Increase above baseline is based on a collection volume of 50–60 ml whole blood with a single pass (except for the cell saver device that uses 450 ml) (Alsousou 2014)

PRP Preparation

Preparation Methods

Autologous PRP can be prepared in a laboratory or an operating theatre or clinic room from anticoagulated blood collected in the immediate pre-therapeutic period. There are several choices of anticoagulants that can be used for blood collection. Trisodium citrate solution is the most widely used anticoagulant (citrate is a weak chelator of calcium that inhibits coagulation and platelet function) with few negative effects on PRP preparation (Alsousou et al. 2009). ACD (acid citrate dextrose) and CPD (citrate phosphate dextrose), with and without adenine (ACD-A and CPD-A), are also effective anticoagulants. The use of ethylenediaminetetraacetic acid (EDTA) is potentially more harmful in the preparation of PRP, and a large number of damaged/activated platelets have been observed during preparation with this material. Therefore it is not usually recommended for PRP preparation.

Traditionally, a relatively pure preparation and good yield of PRP can be easily obtained in a single step by low g centrifugation (170–200 g) of anticoagulated blood for 10 min at room temperature (Harrison et al. 2011a). A consensus of the working party from the Platelet Physiology Subcommittee of the Scientific and Standardisation Committee (SSC) at the International Society on Thrombosis and Haemostasis (ISTH) provides a g value recommendation for platelet preparation (Cattaneo et al. 2013).

For clinical applications there is no such standardisation, and there are three essential main methods available which can rapidly provide a sterile product suitable for clinical application: the (1) gravitational platelet sequestration (GPS) or centrifugation technique, (2) standard cell separators and (3) autologous selective filtration technology (plateletpheresis).

In the GPS method, three layers become evident: the bottom layer comprised of red blood cells (specific gravity = 1.09), the middle layer comprised of platelets and white blood cells (buffy coat, specific gravity = 1.06) and the top plasma layer (specific gravity = 1.03). Various methods to

collect the platelet layer have been developed, but with varying degrees of red and leucocyte contamination (Table 1). Platelet concentration, yield and recovery are also dependent on the centrifugation protocol and collection method (Kevy and Jacobson 2004). One study evaluated the effect of different centrifugal forces and showed that centrifugation at greater than 800 g may actually decrease the amount of releasable TGF-β from the PRP (Landesberg and Glickman 2000).

Standard cell separators and salvage devices generally operate on a full unit of blood. In general, they use continuous flow centrifuge bowls or continuous flow disc separation technology coupled with hard and soft centrifugation steps. Weibrich et al. described a discontinuous technique with a cell separator that produces a fivefold increase in platelet count (Weibrich et al. 2002). The red blood cells and some, or all, of the platelet-poor plasma (PPP) can also be returned to the patient to maintain circulating volume.

Selective filtration technology or plateletpheresis depends on a single-use proprietary filter designed to concentrate platelets from whole blood. The platelets are captured on the filter and are then harvested to provide a platelet-rich concentrate (PRC). Although this technique reduces platelet lysis, which may be induced during centrifugation, platelet fragmentation and activation have also been shown to occur within filtration-based systems (Schoendorfer et al. 1990; Kevy and Jacobson 2004).

In general, most systems do not concentrate the plasma proteins of the coagulation cascade (Kevy and Jacobson 2004). Plasma protein concentrations above baseline can be achieved through secondary ultrafiltration. The autologous growth factor filter (AGF) is a microporous, hollow, cellulose fibre filtration device with a volume of 8 ml and uses PRP as a baseline product. The device filters water after multiple passes of PRP through the filtration device. As much as two-thirds of the aqueous phase is removed by filtration, thus increasing the concentrations of the retained plasma proteins and formed elements (Everts et al. 2006). Evidently each preparation technique will also result in significant differences in yields, concentration, purity, viability and activation status of the isolated platelets. Each of these variables will therefore not only influence the eventual concentrations of the bioactive proteins released from the platelet granules but also affect the eventual clinical efficacy of each PRP preparation (Whitman et al. 1997).

Handling and Activation of PRP

Once the PRP is prepared, it is claimed that it is stable for clinical use, in the anticoagulated state, for 8 h or longer (Marx et al. 1998). Some authors advocate using PRP within no longer than 6 h if it is kept at room temperature (Ho and Chan 1995). However, Sweeney et al. (2006) suggested using a specific method, which included adding low-pH glucose-containing additive solution with $NaHCO_3$ to platelets within ELX bags, to try and keep PRP viable for up to 7 days by lowering the pH to allow sterilisation and provide energy to maintain platelet viability.

PRP must also be activated for the platelets to release their α-granule contents, with the clot providing a vehicle to capture the secreted proteins and maintain their presence at the application site. Activation with bovine thrombin is no longer recommended as this was reported to cause coagulopathy resulting from cross reactivity of anti-bovine factor V antibodies with human factor V (Cmolik et al. 1993; Landesberg et al. 1998). Calcium chloride is therefore a much safer, but slower, alternative and without any risk of prion or pathogen exposure. Platelet activation via this method takes at least 20 min (Anitua et al. 2004). Recently, autologous thrombin preparation kits have been introduced as a safe and faster activation alternative (Man et al. 2001). Furthermore, injecting inactive PRP into collagen rich tissues may also result in direct platelet activation resulting in degranulation (Fufa et al. 2008; Harrison et al. 2011b). Harrison et al. found that thrombin-activated platelets lead to immediate release of TGF-β1 and PDGF-AB, while the collagen-activated PRP clots resulted in 80 % greater cumulative release of TGF-β1, VEGF and PDGF than thrombin over 7 days (Harrison et al. 2011b).

In Vitro and Animal Studies of PRP

Cell and Tissue Culture Studies

PRP influences the migration, proliferation and differentiation of a number of cell types, although the temporal features and molecular basis of this effect remain unclear. Castelnovo et al. (2000) demonstrated an 80-fold enhancement of porcine retinal cell migration 48 h after the application of human platelet suspension, and PRP has been shown to promote the recruitment of circulation-derived cells for tendon healing in vivo (Kajikawa et al. 2008). PRP can also have a mitogenic effect on endothelial cells (Bertrand-Duchesne et al. 2010), bone (Bertoldi et al. 2009), cartilage (Akeda et al. 2006; Ishida et al. 2007), periodontal ligaments (Pantou et al. 2010) and mesenchymal and dental stem cells (Feng et al. 2010; Huang and Wang 2010). However, there are conflicting reports concerning the effects of PRP on cell differentiation. While several studies indicate a positive influence (Bertoldi et al. 2009; Huang and Wang 2010; Lee et al. 2011; Lucarelli et al. 2005), PRP has been shown to inhibit the osteogenic differentiation of bone marrow-derived pre-osteoblasts in a dose-dependent manner (Arpornmaeklong et al. 2004) and has an inhibitory effect

on mesenchymal stem cell differentiation when applied in isolation, but a stimulatory effect with 1,25(OH)2vitamin D3 co-treatment (Feng et al. 2010).

The effect of PRP on cellular activity appears to be highly time and dose dependent. Soffer et al. (2004) showed that while short-term exposure to human platelet lysate (<24 h) promotes bone cell proliferation and chemotaxis, long-term exposure results in decreased levels of alkaline phosphatase and mineral formation. Other studies indicate that PRP stimulates the growth of bone marrow cells for 4–8 days (Van Den Dolder et al. 2006) and that, beyond 6 days, washed platelet solution has a stronger influence on mesenchymal cell proliferation than PRP (Duan et al. 2011). The concentration of PRP also appears to be critical in determining its effect. Some authors suggest that high concentrations adversely affect fibroblast cell viability and proliferation (Choi et al. 2005; Creeper et al. 2009). Cheng et al. demonstrated the significance of both the platelet and plasma protein constituents of PRP as these fail to significantly enhance gene expression of collagen types 1 and 3 in cultured ligament cells when applied in isolation, but do succeed when in combination (Cheng et al. 2010).

Several studies have confirmed the ability of PRP to induce local gene expression. The growth factors TGF-β1 and PDGF appear to play an important role in tendon and ligament regeneration by promoting expression of proteins including collagen and cartilage oligomeric matrix protein (Cheng et al. 2010; Lyras et al. 2010c; Schnabel et al. 2007). Histologically, PRP has been shown to promote gross ligamentisation of tendons (Sanchez et al. 2010) and increase the maximum breaking strength and stiffness of healing tendons in vitro when used with a bone marrow-derived stromal cell-seeded graft (Morizaki et al. 2010). Activation of the Nrf2-antioxidant response element pathway has been implicated in the increase in tenocyte growth due to addition of platelet-released growth factor (Tohidnezhad et al. 2011). Further, Lyras et al. (2010b, 2011) showed that a single application of PRP to the Achilles and patellar tendon defects significantly enhances IGF-1 protein expression and is associated with a faster healing process confirmed by histology (thicker tendons with organised fibre structure). Anitua et al. demonstrated that PRP application results in increased expression and production of VEGF and HGF in cultured tenocytes (Anitua et al. 2005). Other in vitro studies confirmed that the ability of PRP to promote angiogenesis, which is crucial in acute tissue injury repair, may be an important reason for its regenerative effects (De Mos et al. 2008; Lyras et al. 2010a; Lyras et al. 2009).

It is unclear whether the mitogenic effects of PRP are dependent upon the presence of intact platelets or not. Lysed solutions have been shown to have a positive effect on osteogenic activity (Slater et al. 1995) and bone marrow stem cells (Oprea et al. 2003), but other studies also suggest that unviable or damaged platelets after processing may have impaired growth factor secretion (Marx 2001, 2004).

In Vivo Animal Studies

PRP has been shown to promote bone regeneration in rat and rabbit calvarial defects (Mariano et al. 2010), the alveolar sockets of *Cebus apella* monkeys (Pessoa et al. 2009) and porcine tibial metaphyseal defects (Jungbluth et al. 2010), as well as promote the incorporation of mandibular bone grafts in goats (Fennis et al. 2002). However, other studies suggest that PRP has no effect on bone healing and may actually be detrimental when used as a supplement to bone grafts. In a study looking at craniofacial bone repair in rabbits, PRP-treated grafts were associated with diffuse fibrous tissue deposition that inhibited bone formation when compared with untreated grafts (Giovanini et al. 2011). Broggini et al. also showed that PRP alone, or when used in combination with autogenous bone, failed to promote remodelling in rabbit calvaria, whereas autogenous bone without PRP leads to accelerated remodelling (Broggini et al. 2011).

Transected rat Achilles tendons treated with PRP showed increased callous strength and stiffness by 30 % after 1 week (Aspenberg and Virchenko 2004). Similar effects were observed on lesioned equine superficial digital flexor tendons treated with PRP with significantly higher strength at failure, improved collagen network organisation and increased metabolic activity than saline-treated controls (Bosch et al. 2010). Other animal and in vitro studies have also suggested a key role for angiogenesis as a mechanism underlying the regenerative effects of PRP (Anitua et al. 2005; Bir et al. 2011; De Mos et al. 2008; Li et al. 2009; Lyras et al. 2010a). In a porcine model, Hadad et al. showed that adipose-derived stem cells and PRP significantly enhance healing of perfusion-depleted tissue when applied in combination, but not in isolation, lending support to the hypothesis that the growth factors contained in activated PRP have a permissive effect in enabling regeneration (Hadad et al. 2010).

Animal studies have also shown that PRP application improves left ventricular function following ischaemia reperfusion (Mishra et al. 2011), promotes fat graft survival (Nakamura et al. 2010) and enhances the repair of the cartilage (Milano et al. 2010), skin (Derossi et al. 2009), corneal epithelium (Tanidir et al. 2010), liver (Matsuo et al. 2011), intestine (Yamaguchi et al. 2012) and peripheral nerves (Yu et al. 2011).

Clinical Studies of PRP

Many clinical research studies are either predominantly case studies or limited case series. There have been several randomised trials (RCT) investigating the use of PRP, with a recent systematic review and meta-analysis identifying 23 trials in 13 conditions (Sheth et al. 2012). Pathologies studied include arthroplasty, spinal fusion and a range of tendon pathologies. The 2012 review used the GRADE evaluation method and concluded that the quality of evidence is very low. The authors concluded 'the current literature is complicated by a lack of standardization of study protocols, platelet separation techniques, and outcome measures'. However, despite this uncertainty about the evidence, there is increasing clinical use of PRP within clinical practice in many areas with sports medicine being the most popular.

Bone

There are few clinical studies examining the role of PRP in bone healing after orthopaedic trauma (Alsousou et al. 2009; Pietrzak and Eppley 2005). Most of the published studies are related to the use of PRP in oral and cranial surgery. Currently, it is not uncommon to combine the platelet-rich material with autograft, allograft, demineralised bone matrix or other graft material to fill bony defects in the mandible or cranium (Marx et al. 1998).

In small cohort studies, percutaneous injection of autologous platelet–leucocyte-rich gel has been shown to be an effective and less invasive alternative to bone marrow injection in the treatment of delayed bone union (Bielecki et al. 2012) to promote faster fusion in spinal surgery (Hartmann et al. 2010; Lowery et al. 1999) and healing of clavicle fracture nonunion (a single case) (Seijas et al. 2010).

In bony defects, PRP reduces the time taken for bone regeneration (Nagaveni et al. 2010; Rodrigues et al. 2011) and has been used to treat mandibular osteonecrosis resulting from radiotherapy (Scala et al. 2010) or the use of bisphosphonates (Curi et al. 2007). Cenni et al. recently provided a possible biological explanation for this through the demonstration that growth factor release from platelets is maintained within regions of osteonecrosis (Cenni et al. 2011). In one series, researchers were able to measure levels of PDGF and TGF-β in the fracture haematoma of 24 patients who had fresh fractures of the foot and ankle. However, the investigators were unable to detect these same proteins in the nonunion tissue of seven patients presenting with similar fractures. After PRP application of the nonunion during revision surgery, radiographic union was observed by an average of 8.5 weeks (Gandhi et al. 2005).

The use of PRP in maxillofacial surgery is well supported with recently published level 1 and 2 evidence (meta-analysis or randomised controlled trials). However, there is no level 1 or level 2 evidence showing any cortical bone benefits from PRP. There are just a few anecdotal reports on the effect on a mix of corticocancellous bone or a mixture of PRP and bone substitutes.

Wound Healing

As early as 1990, autologous human platelet-derived wound healing factors (HPDWHF) were proposed to regulate wound healing of recalcitrant skin ulcers by promoting the formation of granulation tissue in the early healing phase (Herouy et al. 2000). PRP and other platelet-rich products have been shown to promote healing in a range of cutaneous lesions (Crovetti et al. 2004; Lacci and Dardik 2010; O'connell et al. 2008) and have therefore attracted interest in fields such as plastic surgery where structured approaches to the use of PRP are beginning to emerge (Gentile and Cervelli 2010). In a randomised controlled trial, diabetic foot ulcers treated with PRP gel showed significantly reduced time to healing compared with controls (Driver et al. 2006). While there have been reports of negative results with the use of platelet-derived wound healing factors in ulceration, most notably in a trial by Krupski et al. (1991), more recent studies using autologous PRP have supported its beneficial role in chronic wound healing and provided evidence that it may actually reverse non-healing trends (Frykberg et al. 2010; Lacci and Dardik 2010).

Post-traumatically, PRP promotes ulcer healing (Cervelli et al. 2011a) and faster re-epithelialisation of lower extremity wounds with bone exposure (Cervelli et al. 2011b) and, in combination with hyaluronic acid, can act as an effective scaffold for cellular growth in covering exposed tendons in acute and chronic open wounds of the foot and ankle (Cervelli et al. 2010). In a 5-year study assessing the effect of applying platelet gel to patients presenting with traumatic loss of finger substance, Balbo et al. reported better aesthetic results and a shorter recovery time in the PRP-treated group (Balbo et al. 2010). However, although an early case report suggested that PRP promotes healing even in the presence of infection (Cieslik-Bielecka et al. 2009), recent randomised studies have shown that it fails to actually prevent infection if administered during inguinal surgery (Lawlor et al. 2011) or during wound closure following venous harvesting (Almdahl et al. 2011).

Marck et al. recently performed a systematic review of the use of PRP in burn injury (Marck et al. 2014). They concluded that the literature on the use of PRP in burns is scarce. Despite this growth factors have been shown to be beneficial, and both animal studies and case reports have

shown that PRP can improve healing times post burn injury. Deep dermal burns may also benefit from the pro-haemostatic effects, antimicrobial properties and well-known positive effects of platelets on wound healing. However, patients with large burns also have profound changes to their physiological state including changes in platelet counts observed post injury, and it is unknown how this may affect the quality of autologous platelet preparations (Marck et al. 2013). Also it is largely unknown how PRP may affect long-term scarring given that platelet growth factors such as TGF-β are implicated. Overall they concluded that further research is required to study the potential efficacy of PRP in burns. One of the obvious problems in burn injury is how to apply activated PRP gel evenly over a large surface area of injury while maintaining biological activity.

Use of PRP in Tendon Disorders

In pilot studies, PRP has been shown to reduce pain in patients with chronic tendinopathy (Gaweda et al. 2010; Kon et al. 2009), with a persisting effect for at least 2 years from the time of treatment despite normalisation of VEGF, EGF and CCL2 blood levels after 24 h (Volpi et al. 2010). More recently, in the 2-year follow-up of a double-blind randomised controlled trial comparing PRP with corticosteroid injection for the treatment of chronic lateral epicondylitis, Gosens et al. reported significantly increased function and reduced pain in the PRP-treated group compared to those treated with steroids (Gosens et al. 2011). The benefit of PRP as a second-line, surgery-sparing therapy in the treatment of elbow tendinopathy resistant to physical therapy has been further demonstrated in a prospective randomised trial reported earlier this year (Creaney et al. 2011). Creaney et al. study showed that injecting PRP in chronic tendinopathy reduced the need for surgery from 20 to 10 % when compared with autologous blood injection alone.

Despite the evidence that PRP may be effective in treating tendinopathy, there is also a growing body of evidence to the contrary. The most significant trial in this regard was a double-blind randomised controlled trial carried out by De Vos et al. which showed no difference in pain or function in patients with chronic Achilles tendinopathy treated with PRP compared with those treated with saline (De Vos et al. 2010b). A recent study identified no significant difference in the degree of neovascularisation or tendon structure (using colour Doppler ultrasonography and ultrasonic tissue characterisation) in patients with chronic Achilles tendinopathy treated with PRP compared with those receiving a saline placebo (De Vos et al. 2011). Another application of PRP that has gained significant attention is its use in anterior cruciate ligament (ACL) reconstruction using hamstring tendon grafting following traumatic injury. In a randomised, double-blind trial, Vogrin et al. used contrast-enhanced MRI to demonstrate that ACL grafts treated with platelet gel had a significantly higher level of vascularisation at the osteoligamentous interface 4–6 weeks postoperatively compared to control grafts (Vogrin et al. 2010b). PRP gel has also been shown to reduce the time taken for a homogenous appearance on MRI following ACL reconstruction by 48 % (Radice et al. 2010), although contrary to this Silva and Sampaio demonstrated a failure of PRP to affect the signal intensity of the fibrous interzone in femoral tunnels following grafting (Silva and Sampaio 2009). Functionally, the application of platelet gel to grafts has been shown to significantly improve anteroposterior knee stability following surgery (Vogrin et al. 2010a).

With regard to the use of PRP in treating rotator cuff tears, a pilot study of patients undergoing arthroscopic rotator cuff repair showed that intraoperative PRP administration was associated with significantly increased function and reduced pain at 6, 12 and 24 months postoperatively with no adverse events (Randelli et al. 2008). However, a recent randomised controlled trial demonstrated that platelet-rich fibrin matrix has no benefit in enhancing healing of small- and medium-sized rotator cuff tears (Castricini et al. 2011): as with tendinopathy, the consensus in the literature is that further trials are needed to determine whether neutral results regarding the benefit of platelet-rich mediums indicate a lack of benefit from having additional growth factors in certain healing environments or whether they reflect suboptimal preparation and administration techniques (Engebretsen et al. 2010; Hamilton et al. 2010). Two recent reviews conclude that the poor methodological quality of trials assessing the use of PRP in treating chronic tendinopathy means the evidence supporting its effectiveness is limited and thus insufficient to recommend its routine clinical use (De Vos et al. 2010a; Kampa and Connell 2010).

A thorough literature search of the application of PRP in Achilles tendon rupture in both animal and human studies was recently conducted. Nine studies present results for the use of platelets in Achilles tendon rupture treatment, seven from animal experiments and two from human trials (Sanchez et al. 2007; Schepull et al. 2011). Six of the animal studies used a rat model, and one used an ovine model. All animal studies, using biomechanical and histological assessments, are in agreement showing a beneficial effect of platelets. Only two human studies have tested the effect of platelets on the treatment of Achilles tendon rupture based on imaging techniques and clinical results. Sanchez et al. (2007) found positive effects in a group of 12 athletes treated with PRP-augmented suture repair at 32 months in a case–control study. In ultrasonographic assessment, there was less tendon thickening and higher concentrations of TFG-beta and other growth factors, and patients regained range of

motion faster and returned to sports (gentle running) earlier. In a randomised study of 30 patients, Schepull et al. (2011) found no effect of platelets on radioisometrical tendon contraction or clinical outcomes. Both of these underpowered clinical studies used PRP as an adjunct to open surgical repair, and this may have obscured any effect of PRP on healing.

There are three other studies that report on the use of platelets in human Achilles tendinopathy although the results of these trials have little bearing on Achilles tendon rupture, which has a very different pathology, regenerative properties and treatment pathways. Therefore there is only one underpowered randomised clinical trial that has assessed PRP in Achilles tendon rupture (Sanchez et al. 2007; Schepull et al. 2011), and the authors of that trial recognised a limitation in their platelet preparation technique and storage (of up to 20 h) resulted in only a 20 % release of growth factors from the platelets. In our own 20-patient pilot study, we achieved an average of 69 % growth factor content release after stimulation.

Recently published systematic reviews (Sadoghi et al. 2012; Taylor et al. 2011) have concluded that there are encouraging signs that PRP could be developed as an effective therapy. Sadoghi et al. (2012) concluded that there is evidence in support of a positive effect of platelet concentrates in the treatment of Achilles tendon ruptures in vivo in animal models and human application, consistent with a medium- to large-sized effect. This effect is most likely attributed to enhanced scar tissue maturation. In another systematic review, Taylor et al. (2011) concluded that PRP use in tendon and ligament injuries has several potential advantages, including faster recovery and, possibly, a reduction in recurrence, with no adverse reactions described. One ongoing randomised trial is studying the effect of PRP on ruptured Achilles tendon (Anonymous 2015); the PATH-2 trial will study the efficacy and mechanism of PRP in pragmatic settings which include clinical, histological and laboratory outcome measures, as well as measurement of platelet quality, purity and growth factor content (Anonymous 2015).

Potential Adverse Risks of PRP

PRP is prepared from autologous blood; therefore it is inherently safe, and any concerns of disease transmission such as HIV, hepatitis or Creutzfeldt–Jakob disease or immunogenic reactions that exist with allograft or xenograft preparations are eliminated (Alsousou et al. 2009). However, using bovine thrombin preparations, which contain bovine factor V to activate the platelets, may lead to immunogenic reactions. The systemic use of bovine thrombin in cardiovascular surgery to promote clotting has been reported to be associated with coagulopathies resulting from cross reactivity of anti-bovine factor V antibodies with human factor V (Cmolik et al. 1993; Landesberg et al. 1998). The bovine thrombin preparations used in those reported cases were high dose (>10,000 units) and were applied directly onto open wounds where absorption into systemic circulation is certain. There have been no similar reports since 1997 due to the use of highly purified bovine thrombin. Conversely, the very small dose of bovine thrombin (<200 units) used to activate PRP before application will be consumed during clot formation and digested by macrophages. Hence, bovine thrombin-activated PRP is unlikely to produce anti-factor V antibodies.

The use of bovine thrombin for activation is therefore now avoided in modern preparation techniques. Some authors proposed that PRP gel formation can be performed only with the addition of calcium chloride, but this usually takes longer to complete (Marlovits et al. 2004). An effective alternative is thrombin receptor agonist peptide (TRAP) that mimics the effect of thrombin on PAR-1 receptors. Most current techniques prefer to use autologous thrombin or calcium chloride to activate PRP.

To date, there is no compelling evidence of any systemic complications of local PRP injection. Furthermore, there are no scientific reports suggesting potential cause–effect relationships between growth factors present in PRP and an increased risk of carcinogenesis. This may be because of the limited need for PRP injections (as PRP is not chronically administered) and because of the short in vivo half-lives and local bioavailability of growth factors produced by PRP.

Clinical Trials

Based on the inconclusive results in the current literature, the authors cannot provide solid evidence in favour of the clinical application of PRP. However, because the majority of the reviewed clinical trials reported encouraging outcomes, further controlled clinical trials are warranted to elucidate where PRP should be used.

However, clinical trials should include a sufficient number of patients and a proper design (randomised controlled trials), including a test group similar to control, except for the application of PRP. Also, proper description of the PRP procurement techniques must be included, including the amount of blood collected, baseline number of platelets, yield, purity, quality and growth factor content of PRP obtained, contaminating cells, concentration factor of platelets above baseline, coagulation promoters, doses and mixing ratio to PRP.

Clinical Study Design

Given the rudimentary knowledge of the mechanism of action of PRP in clinical settings and the limitations of current studies, it is important that any future clinical trial should be carefully designed not only with adequate power to accurately determine the effect of PRP on any particular tendon disorder but also to use disease-specific outcome tools. This has been highlighted by several authors (De Vos et al. 2010a; Maffulli and Del Buono 2012), who have shown that the imprecision in effect size estimates from underpowered studies investigating the use of PRP has led to unsafe conclusions. A study powered to determine if there are clinically important effects while furthering the understanding of PRP mechanism in clinical settings would add significantly to our understanding of the appropriate clinically used PRP. In a consensus paper published recently (Engebretsen et al. 2010), a panel of experts listed important elements of clinical trials of PRP: (1) RCT design, (2) clear inclusion/exclusion criteria, (3) homogenous study population or stratification of variables, (4) standardised clinical assessment, (5) validated PRP production method and delivery, (6) robust outcome measures and (7) standardised post-treatment follow-up protocol.

Currently, we are only aware of one ongoing randomised clinical trial that includes all the above and is adequately powered to measure the efficacy of PRP in clinical settings; the PATH-2 trial has started recruiting patients with acute Achilles tendon rupture and is due to finish in 2018 (Anonymous 2015).

PRP Classification System

'Not all PRP is in fact PRP'

In the field of platelet concentrates, most products are termed PRP. Unfortunately, this term is very general and incomplete, leading to confusion in the scientific literature and misleading conclusions. Currently, there are more than 12 platelet concentrate products available commercially, and although they have varying preparation methods and components (Table 1), they are all labelled PRP.

Accurate use of the terminology to reflect the product properties is required in both clinical and scientific applications. The terminology must be simple, accurate and pragmatic. It also must avoid commercial interests and thus remain scientifically homogeneous (Dohan Ehrenfest et al. 2012). Clinically prepared PRP is in fact a term that includes a wide range of products that have varying biological components (Dohan Ehrenfest et al. 2009). Most of these products have varying concentrations of blood cells (platelets, leucocytes and erythrocytes) and microvesicles. Two authors have tried to classify PRP products according to their biological content, which are described below.

Dohan Ehrenfest et al. (2009) classified the different platelet concentrates into four categories, depending on their leucocyte and fibrin content: pure platelet-rich plasma (P-PRP), leucocyte- and platelet-rich plasma (L-PRP), pure platelet-rich fibrin (P-PRF) and leucocyte- and platelet-rich fibrin (L-PRF) (Table 2). Yet, Mishra et al. (Mishra et al. 2012) used the presence of leucocytes and the method of activation to classify PRP into four types (Table 3).

In addition to the aforementioned factors, platelets are very sensitive to in vitro manipulation and can be easily activated during preparation methods leading to the premature release of their bioactive contents before application to the targeted tissues (Alsousou et al. 2013, 2009; Harrison 2004). Therefore details of the preparation method and ultimate platelet quality (e.g. concentration, viability, activation status and growth factor content) may also be important to potentially stratify any clinical effects.

The authors of this chapter suggest a new classification system, which incorporates the above two classification systems and includes the preparation method technique. This new system will use a combination of numeric and alphabetical characters to identify the class of PRP product. In addition to the two identifiers in the Ehrenfest and Mishra classifications, leucocytes and fibrin, our proposed new system will also include further identifiers activation, platelet concentration and preparation method categories.

Table 2 Ehrenfest classification system of the main available PRP products (Dohan Ehrenfest et al. 2009)

PRP class	Leucocytes	Fibrin content
P-PRP	No	Low
L-PRP	Yes	Low
P-PRF	No	High
L-PRF	Yes	High

Table 3 Mishra classification is divided into four types (1–4) and two subtypes (A and B) (Mishra et al. 2012)

	White cells	Activation	Platelet concentration
Type 1	Increased	No activation	A >5× B <5×
Type 2	Increased	Activated	A >5× B <5×
Type 3	Minimal or no WBC	No activation	A >5× B <5×
Type 4	Minimal or no WBC	Activation	A >5× B <5×

Table 4 The modified PRP classification system. The subcategories of activation, platelet concentration and preparation technique are added after the main class (e.g. L-PRP IB1) (Alsousou 2014)

Class	Leucocytes	Fibrin	Activation	Platelet concentration	Preparation category
P-PRP	–	Low	I II	A B C	1 2 3
L-PRP	+	Low	I II	A B C	1 2 3
P-PRF	–	High	I II	A B C	1 2 3
L-PRF	+	High	I II	A B C	1 2 3

Activation is divided into two subcategories:

I for the use of activation
II for PRP application without activator

Unlike the Mishra classification, platelet concentrates are subdivided into three categories (A, B and C) based on the range of platelet counts in the samples. This is in line with evidence presented previously in this chapter (Weibrich et al. 2004), which states that the most effective platelet count range is 900–1700 $\times$ 10^3/μl. The three categories are:

A. Platelet count $<900 \times 10^3$ /μl
B. Platelet count 900–1700 $\times$ 10^3/μl
C. Platelet count $>1700 \times 10^3$/μl

Furthermore, the preparation methods are classified into three categories:

1. The gravitational platelet sequestration (GPS) technique
2. Standard cell separators
3. Autologous selective filtration technology (plateletpheresis)

This modified classification is presented in Table 4. For example, the PRP used in conducting our PATH-2 trials will be classified as L-PRP IB1, which indicates that this PRP is prepared using the GPS method, which contained leucocytes with a platelet count range of 900–1700, and denotes that the platelets have been activated before application.

Conclusion

The role of PRP in tissue regeneration and its clinical application are a fast-developing field. Although, the effect of PRP on cell lines and tissues has been studied both in vitro and in animal models, clinical applications are still lacking the level of evidence from purposely designed randomised controlled trials. Further controlled clinical trials to elucidate the effects of PRP in pragmatic settings are warranted that need to take into account the quality control of the various PRP preparations utilised to ensure that optimal relationship between the product and outcome is established. Further studies into the mechanism of PRP tissue regeneration may also help elucidate the optimum combination of bioactive factors required to achieve best clinical efficacy.

Take-Home Messages

- The regenerative effect of PRP exerted by producing a local environment for tissue regeneration which is rich in growth factors and other cytokines has been supported by in vitro and animal studies.
- The clinical application of autologous PRP in regenerative medicine has become very popular in many areas of medicine. Despite this, there is a lack of adequately powered trials to provide solid evidence for its clinical use.
- Clinical preparations of PRP are heterogeneous and are often poorly standardised and defined.
- Future trials should be adequately powered to include a sufficient number of patients and controls and include a full description of the PRP preparation methodology utilised as well as the yield, purity, quality and growth factor content of the PRP used.
- We present a new classification system of PRP taking into account the actual content (e.g. with leucocytes and fibrin), the activation methodology, the platelet concentration obtained and the preparation method used.
- Further clinical trials in this area are warranted taking into account the quality of the various PRP preparations utilised to enable a link between sample quality and clinical outcomes, which is also to be established.

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Therapeutic Use of Thrombopoietin Receptor Agonists

Michael D. Tarantino and Sarah Chalmers

Abstract

Discoveries throughout the twentieth century have forged our understanding of platelet production and including the variables involved in regulating the growth and maturation of megakaryocytes (Kuter, Br J Haematol 165:248–58, 2014). Thrombopoietin (TPO) is the premier growth factor involved in platelet production. It was first discovered in the early 1990s. TPO binds to the thrombopoietin receptor (TPOr, also termed c-mpl) on platelets, megakaryocytes, and pluripotent stem cells leading to inhibition of apoptosis of stem cells and megakaryocytes; increased megakaryocyte number, size, and ploidy; increased rate of megakaryocyte maturation and platelet count; and decreased platelet threshold for activation by ADP and collagen. Since the discovery of TPO, several TPO or TPO mimetic medications have been developed. Initially, two recombinant human thrombopoietins were developed; however, production was stopped following the discovery of autoantibodies in healthy platelet donors to both the drug and endogenous TPO after trialing the medication. Since then nonrecombinant peptide and non-peptide thrombopoietin receptor agonists (TRAs) have passed several trials in various thrombocytopenia-related disease processes including cirrhosis and/or chronic hepatitis C infection, aplastic anemia, chemotherapy-induced thrombocytopenia (CIT), Wiskott-Aldrich syndrome (WAS), MYH-9 disorders, and, primarily, immune thrombocytopenia (ITP). This chapter will highlight the therapeutic uses of TRAs in thrombocytopenic disorders.

Thrombopoietin

Thrombopoietin is the primary regulator of thrombopoiesis. Throughout the twentieth century, several discoveries lead to the isolation of TPO and the understanding of its role in thrombopoiesis. Megakaryocytes were discovered as the platelet-producing cells in the bone marrow by Wright in 1906 (Wright 1906). In 1958, Professor Endre Kelemen first introduced the concept of TPO as a regulatory factor in thrombopoiesis (Kelemen et al. 1958). Kelemen confirmed his conclusion when he demonstrated a rise in platelet count after injection of sera from patients with myeloproliferative disease into thrombocytopenic mice and humans (Kelemen et al. 1963). Following these discoveries, it would be more than 30 years until TPO was isolated.

In 1994, several research groups simultaneously published results on the successful purification of TPO (Sohma et al. 1994; Lok et al. 1994; Kaushansky 1994; Kato et al. 1995; Bartley et al. 1994; Kuter et al. 1994). Following its purification, the discovery of thrombopoietin as the primary regulator of thrombopoiesis was demonstrated in TPOr or TPO gene knockout mice. The recipient mice had a profound decrease in platelet production and subsequently a precipitous drop in platelet count, proving that TPO stimulates thrombopoiesis (Alexander et al. 1996; Gurney et al. 1994; De Sauvage et al.

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1996). TPO is a 353 amino acid precursor protein possibly produced in several organs but predominately in the liver parenchyma. It has two distinct domains separated by a potential cleavage site. The N-terminal region contains the receptor-binding domain and consists of a four-helix structure with 23 % amino acid homology to erythropoietin (EPO). The C-terminus serves to prolong the half-life of the molecule as well as act as a molecular chaperone in the folding of the four-helix structure (Kaushansky 2015).

The TPOr is a dimeric transmembrane receptor found on pluripotent stem cells, immature cells from all cell lines, mature megakaryocytes, and platelets. The binding of TPO to TPOr activates various signal transduction pathways including JAK, STAT, and MAP kinase. The effects of this activation lead to inhibition of hematopoietic stem cells (HSCs) and megakaryocyte apoptosis; HSC differentiation; megakaryocyte maturation; increased megakaryocyte ploidy, number, and size; lower threshold for platelet activation; and involution of the TPO-TPOr complex (Kuter et al. 1994; Li et al. 1999; Zauli et al. 1997).

Early Production of Thrombopoietin Receptor Agonists

Thrombopoietin receptor agonists (TRAs) were first developed in the 1990s. Two recombinant TRAs were initially trialed. One was a recombinant full-length human thrombopoietin (rhTPO) analogous to endogenous TPO. The other was a truncated version of TPO consisting of an analogue of the binding domain of TPO attached to a polyethylene glycol molecule and termed polyethylene glycol (PEG)-ylated-recombinant human megakaryocyte growth and development factor. They were initially trialed for the treatment of chemotherapy-induced thrombocytopenia, myelodysplastic syndrome (MDS), and immune thrombocytopenia with good success (Basser 1996). However, when studied for the use of increasing platelet yields in healthy autologous platelet donors, a small portion of the study group developed neutralizing antibodies to the recombinant TPO as well as endogenous TPO (Li 2001). Production of both recombinant TRAs was discontinued. All study participants eventually recovered. Around the same time, two nonhomologous TRAs, later dubbed "the second-generation" TRAs, were in development.

Second-Generation Thrombopoietin Receptor Agonists (TRAs)

Romiplostim

Romiplostim (Nplate®; Amgen Corporation, California, USA) is a weekly administered parenteral TRA composed of a peptide pair inserted into the arm of an immunoglobulin G heavy-chain molecule. It has no sequence homology to endogenous TPO and therefore is predicted to impart a low risk of cross-reactive neutralizing antibodies. The molecular profile of romiplostim is shown in Table 1. Similar to other IgG fusion molecules, romiplostim binds to the FcRn receptor on endothelial and other cells expressing FcRn and is recycled back into the circulation, producing a circulatory half-life of some 120–140 h (Wang et al. 2004). Romiplostim binds to the extracellular domain of the TPOr, stimulating megakaryocyte growth and platelet production, and lowers the platelet activation threshold (Broudy 2004; Cwirla 1997; Gardiner 2010) (see Fig. 1). Romiplostim is a competitive agonist with endogenous TPO but has not been shown to impair the proliferation response to endogenous TPO. Romiplostim may stimulate alternative pathways leading to an enhanced effect of endogenous TPO in conjunction with romiplostim (Broudy 2004). Romiplostim increases platelet count in a dose-dependent fashion (Bussel et al. 2006). The median peak concentration is achieved in 14 h after a single subcutaneous dose, although the serum concentration is highly variable between patients and does not appear to correlate with dose. The biological half-life of romiplostim is 1–34 days with a median of 3.5 days. The recommended starting dose is 1 mcg/kg actual body weight, given subcutaneously, with dose titration in increments of 1 mcg/kg weekly until a

Table 1 Molecular characteristics of the TRAs

Name	IUPAC name	Class	Formula	Molecular weight
Romiplostim[a]	L-Methionyl[human immunoglobulin heavy constant gamma 1 (227 C terminal residues) peptide (Fc fragment)] fusion protein with 41 amino acid peptide, (7 7′:10,10′) bis-disulfide dimer	Peptibody	$C_{2634}H_{4086}N_{722}O_{790}S_{18}$	~60 kDa
Eltrombopag[b]	3-[(5E)-5-[[2-(3,4-Dimethylphenyl)-5-methyl-3-oxo-1H-pyrazol-4-yl] hydrazinylidene]-6-oxocyclohexa-1,3-dien-1-yl]benzoic acid	Small molecule	$C_{25}H_{22}N_4O_4$	442.4 g/mol
Avatrombopag[c]	1-[3-Chloro-5-[[4-(4-chlorothiophen-2-yl)-5-(4-cyclohexylpiperazin-1-yl)-1,3-thiazol-2-yl]carbamoyl]pyridin-2-yl]piperidine-4-carboxylic acid	Small molecule	$C_{29}H_{34}C_{l2}N_6O_3S_2$	649.6 g/mol

[a]http://www.drugbank.ca/drugs/DB05332, last accessed 29 December 2015
[b]http://www.drugbank.ca/drugs/DB06210, last accessed 29 December 2015
[c]http://pubchem.ncbi.nlm.nih.gov/compound/9918581#section=Top, last accessed 29 December 2015

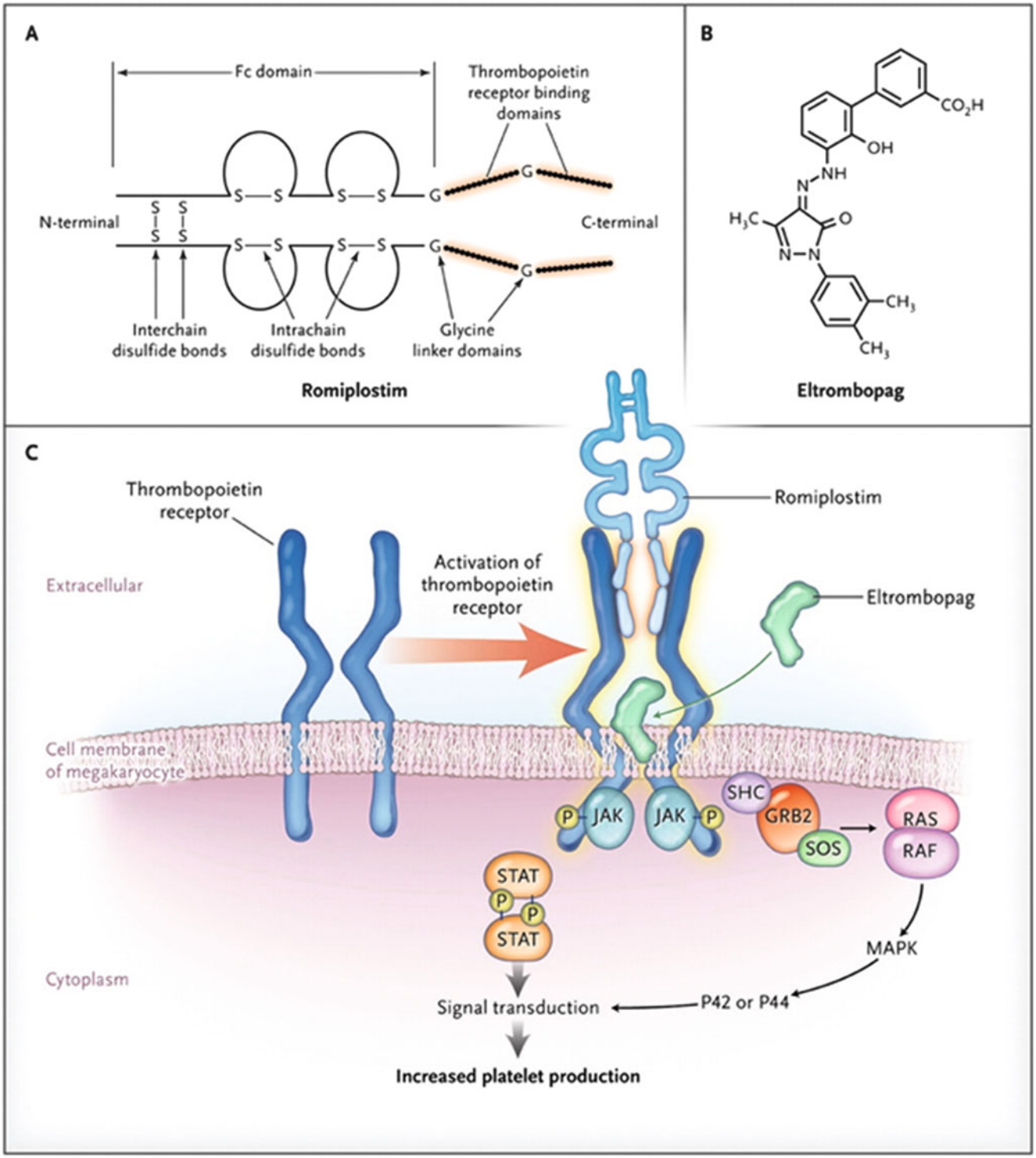

Fig. 1 Schematic of the molecular structure of (**a**) romiplostim and (**b**) eltrombopag. The binding sites on TPOr for romiplostim, to the extracellular domain, and eltrombopag, to the transmembrane domain, are shown in (**c**). From Imbach P, Crowther M. (2011) Thrombopoietin-receptor agonists for primary immune thrombocytopenia. N Engl J Med 365:734–41. Copyright © (2011) Massachusetts Medical Society. Reprinted with permission from Massachusetts Medical Society

platelet count $>50 \times 10^9$/L is achieved or until maximum dose of 10 mcg/kg is reached. It has been suggested that the initial starting dose of 1 mcg/kg may be inadequate leading to poor initial response and delay in effective dose. In particular, the initial dose may be too low for patients with more severe refractory disease and some advocate for a higher initial starting dose in this patient population (Mitchell and Bussel 2015). Discontinuation is recommended if the platelet count does not exceed $>50 \times 10^9$/L after 4 weeks of treatment with maximum dosing at 10 mcg/kg/week. Weekly monitoring of platelet count is recommended until the platelet count remains stable for at least four weeks, with monthly monitoring thereafter. No drug interactions have been identified, and romiplostim is safe to use in combination with other thrombocytopenia treatment regimens such as corticosteroids or immune globulin.

Eltrombopag

Eltrombopag (Promacta®, Revolade®; Novartis, Malmo, Sweden) is an oral synthetic non-peptide small molecule TRA. It binds to the transmembrane domain of the TPOr at residue 499 leading to differentiation of hematopoietic stem cells into megakaryocyte progenitor cells, stimulates the growth of megakaryocytes, and hence increases the platelet count (see Fig. 1). The molecular profile of eltrombopag is shown in Table 1. In vitro studies have also demonstrated stimulation of multi-lineage hematopoiesis (Erickson-Miller et al. 2005, 2009). The binding site of eltrombopag differs from the binding site of endogenous TPO, therefore potentially allowing for an additive or synergistic effect (Erickson-Miller et al. 2009). Eltrombopag has several unique features, different from recombinant TPOs. It binds to the TPO receptor in the transmembrane domain. Its smaller molecular size theoretically reduces the risk of immunogenicity and allows for oral administration. It has species specificity and is only effective in species with a histidine at the 499 residue of the TPOr (humans and chimpanzees only) (Erickson-Miller et al. 2009; Sellers et al. 2004). Eltrombopag reaches peak concentration in 2–6 h after oral administration and has an oral absorption rate of approximately 52 %. The half-life of eltrombopag is 21–32 h. Greater than 99 % is bound by human plasma proteins. Eltrombopag is metabolized in the liver via CYP1A2, CYP2C8, and UDP-glucoronyltransferase1A1 and 1A3. Eltrombopag and its metabolites have an inhibitory effect on several enzymes of metabolism, specifically OATP1B1 and BRCP, leading to drug-drug interactions with many commonly used medications such as statins, antihyperglycemic agents, angiotensin receptor blockers, imatinib, and methotrexate. It has also been shown to be a chelating agent, and it is therefore recommended that it be taken 4 h before or after ingestion of foods or medications (such as antacids) high in calcium or polyvalent cations. On average, the platelet count increases in the first 1–2 weeks. While eltrombopag has been shown to increase platelet count, it does not appear to have an effect on platelet function (Haselboeck 2013).

The recommended starting dose is 25–50 mg (dependent upon underlying disease state causing the thrombocytopenia) by mouth per day, and the maximum recommended dose is 75 mg/day in patients with thrombocytopenia secondary to chronic ITP, 100 mg/day in patients receiving treatment for thrombocytopenia related to hepatitis C, and 150 mg/day in patients receiving treatment for severe refractory aplastic anemia (Table 2). After initiating eltrombopag, the dose can be increased by 25–50 mg every 2 weeks and titrated to a goal platelet count of $>50 \times 10^9$/L (Jenkins et al. 2007). Lower initial doses are recommended in patients of East Asian descent (due to a 33 % lower clearance rate) and those with hepatic failure (Katsutani et al. 2013). Weekly monitoring of the platelet count is recommended until platelet count has remained stable for at least 4 weeks and then monthly thereafter. In addition, a baseline complete metabolic panel (CMP) including liver function studies should be obtained prior to initiation of treatment and weekly until the platelet count has remained stable for at least 4 weeks. Serum bilirubin as well as alanine aminotransferase concentrations should be closely monitored. Liver function should be assessed by monthly CMPs thereafter.

Table 2 Administration and dosing of the TRAs

Name	Manufacturer	Route of administration	Approved starting dose
Romiplostim[a]	Amgen	Subcutaneous	1 mcg/kg
Eltrombopag[b]	Novartis	Oral	50 mg[c]
Avatrombopag[d]	Eisai	Oral	NA

[a]Prescribing information for romiplostim: http://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=c45f9a58-37c1-4f76-8e36-97d38c577037, last accessed 29 December 2015
[b]Prescribing information for eltrombopag: http://dailymed.nlm.nih.gov/dailymed/drugInfo.cfm?setid=616224ff-a925-4b38-9ca2-00fbf669380f, last accessed 29 December 2015
[c]Starting dose for patients of Asian ancestry and children 1 to ≤6 years of age is 25 mg/day
[d]Not yet approved in any country (as of December 2015)

TRAs as a Treatment for Immune Thrombocytopenia

Primary immune thrombocytopenia (ITP) is an autoimmune disorder that leads to peripheral destruction, as well as a decreased production of platelets. In ITP, there are a normal or increased number of megakaryocytes and an increase in cell cycling of platelet progenitor cells. However, the platelet turnover is inappropriately normal or decreased suggesting that the effect of the autoantibodies is toward the end of the thrombopoietic cycle (McMillan et al. 1978; Ballem et al. 1987). In children, most often, the disease is self-limited and resolves within 6 months. Often, "watchful waiting" is the chosen treatment in this population though significant bleeding can occur and certainly warrants drug treatment. In adults, ITP is more often a chronic illness and treatment is usually required for platelet count $<30 \times 10^9$/L. Serum concentrations of TPO levels are normal to slightly low in ITP, likely due to removal of platelet-bound TPO as the platelet is cleared in the reticuloendothelial system (Kuter et al. 1994).

The most extensive clinical research in TRAs has been in the treatment of chronic ITP. Both TRAs have demonstrated overall response rates at or above 80 % of study participants (Kuter et al. 2008; Bussel et al. 2007). Response rates have been at least equal to treatment with first-line therapy, and some studies suggest that the response rate is higher (Zeng 2011). Based on placebo-controlled trials, but not head-to-head comparison trials, efficacy rates of romiplostim and eltrombopag have been similar in that both TRAs increase platelet count, decrease rate of bleeding, decrease the need for rescue therapy, decrease splenectomy rates, increase rate of sustained platelet response, and improve quality of life (Bussel et al. 2006; Kuter et al. 2010; Kuter et al. 2013; Khellaf 2011; George 2009; Michel 2011; Gernsheimer 2010; Bussel et al. 2007, 2009; Cheng et al. 2011).

Sustained remission following cessation of romiplostim has also been seen in a portion of study participants with chronic ITP, independent of previous therapy, splenectomy, and disease duration (Červinek et al. 2015). This may be due to induction of tolerance to platelet antigens (Ghadaki 2013; Mahévas 2014). Clinical trials with eltrombopag for the treatment of ITP have yielded similar results with a (on treatment) sustained response rate near 90 % and peak response rate occurring at approximately day 21 (Saleh et al. 2013).

Recent clinical trials of the TRA use in children with persistent or chronic ITP suggest that romiplostim and eltrombopag are useful therapeutic options, to date without reports of the adverse event signals of thrombosis or marrow reticulin accumulation.

In a phase II dose escalation clinical trial of 22 splenectomized and unsplenectomized children with ITP treated with romiplostim vs. placebo, 88 % vs. 0 of study participants achieved a platelet count $>50 \times 10^9$/L for at least two consecutive weeks with a reduction in clinically relevant bleeding and decreased need for rescue therapy. The median time to the goal platelet count was 7 weeks with an effective dose of 5 μg/kg. Splenectomized patients required a slightly higher dose (Bussel et al. 2011).

In another randomized controlled study of 18 children with chronic ITP, nearly 92 % of patients treated with romiplostim achieved the platelet count target of $>50 \times 10^9$/L by the fifth week. The median weekly dose required to reach this target platelet count was again 5 μg/kg. Those in the treatment arm also had a decreased need for rescue therapy (8 vs. 33 %). Participants in the treatment arm had a significant decrease in bleeding severity. No patients developed thrombocytosis and rebound severe thrombocytopenia or had BM fibrosis at 18 week follow-up (Elalfy et al. 2011).

In a retrospective study of 54 children with ITP treated with either romiplostim or eltrombopag for 6–53 months, 82 % reached the target platelet count of $>50 \times 10^9$/L and $\geq 20 \times 10^9$/L above baseline for at least two consecutive weeks (85 % with romiplostim and 75 % with eltrombopag). Forty-eight percent of patients had at least 50 % of their platelet counts within target range. One patient treated with eltrombopag developed a DVT, however, at the site of an ankle fracture. Forty-four patients underwent bone marrow evaluation and none had histology beyond MF-1 (Ramaswamy et al. 2014).

In the PETIT study, a multicenter randomized controlled study which evaluated children with chronic ITP treated with eltrombopag vs. placebo, 62 vs. 32 % of children achieved at least one platelet count $>50 \times 10^9$/L within the first 6 weeks of treatment. No thrombotic events or malignancy were seen. Three percent of participants had to discontinue treatment due to elevated alanine aminotransferase (Bussel et al. 2015).

In a follow-up to PETIT, PETIT2, 63 children with chronic ITP received eltrombopag. Forty percent achieved the primary platelet outcome, 80 % had any platelet response, fewer in the treatment group had clinically relevant bleeding, and two withdrew because of increased liver aminotransferases. No deaths, malignancies, or thromboses occurred during the trial (Grainger et al. 2015):

Preliminary results from a 6-month phase III randomized placebo-controlled trial of romiplostim in 62 children with ITP lasting longer than 6 months revealed similar results with a durable platelet response that was 52 % overall but a slightly lesser rate in 1–6-year-olds. Rates of serious adverse events (AEs) were 5·3 % (1/19) for placebo and 23·8 % (10/42) for romiplostim. No malignancies or thrombotic events occurred during the study period (Tarantino et al. 2015a, b).

Sixty-five pediatric patients from three previous studies received romiplostim in an open-label trial for up to 269 weeks (ongoing at the time of this printing). Three patients in this study achieved remission (platelet counts $\geq 50 \times 10^9$/L for 24 weeks with no ITP treatments). No new adverse event signals were observed. Again, no thrombotic events or malignancies were reported (Tarantino et al. 2015b).

Several studies have demonstrated efficacy in switching from eltrombopag to romiplostim and from romiplostim to eltrombopag for the treatment of chronic ITP. Despite platelet counts of <50 K $\times 10^9$/L at the time of the switch, many achieve platelet counts >100 K $\times 10^9$/L after switching (Kuter 2015; Khellaf 2013; González-Porras 2015). The reason for this response remains unknown but it may be due to the biological action of each in the TPO binding site and/or intracellular signaling (Khellaf 2013).

Other Thrombopoietin Receptor Agonists

Avatrombopag (AKR501, Eisai, Woodcliff Lake, NJ, USA) is an oral non-peptide TRA that is currently in development. It increases the platelet count in a dose-dependent fashion and has a half-life of approximately 16 h. It is similar to eltrombopag in the following ways: (1) it binds to the transmembrane portion of the TPOr, (2) it stimulates megakaryocytopoiesis at multiple stages including differentiation of progenitor cells, (3) it stimulates hematopoietic progenitor cell proliferation, (4) it has an additive effect on platelet count with endogenous TPO, and (5) it appears to have species specificity and is effective in humans and chimpanzees only. The molecular profile of avatrombopag is shown in Table 1. Avatrombopag increases the platelet count at a lower dose than is required with eltrombopag and does not appear to accumulate in the body (Fukushima-Shintani et al. 2008, 2009; Abe et al. 2011). Avatrombopag has predominately been studied in the treatment of chronic ITP. In adults with chronic ITP, studies have demonstrated a response rate (platelet count $>50 \times 10^9$) of 80 % with 20 mg daily dosing, and in the extension study, the durable response rate was 53 % over a 14-week period. In the extension study, 36 % of nonresponders from the randomized study achieved a durable response. Adverse events were similar to profiles seen with other TRAs (Bussel et al. 2014a, b). A phase II trial of 130 patients with thrombocytopenia secondary to chronic hepatitis C undergoing elective procedure demonstrated a 48 % increase in platelet count $>20 \times 10^9$/L and at least one platelet count $>50 \times 10^9$/L. Treatment was, again, well tolerated. No novel adverse events were identified, although one patient in the treatment group did have portal vein thrombosis (Terrault et al. 2014). Avatrombopag remains under investigation and, to date, has not been approved for treatment of any disease processes.

Adverse Effects of TRAs in Patients with ITP (Table 3)

TRAs are generally well-tolerated medications. The most common side effects among patients are mild-moderate in degree, similar in kind and degree across disease states treated with TRAs, and typically not leading to cessation of treatment (Bussel et al. 2006; Bussel et al. 2007; Kuter et al. 2008; Bussel et al. 2009; Kuter et al. 2010, Khellaf 2011, Cheng et al. 2011; Olnes et al. 2012; Saleh et al. 2013; Afdhal et al. 2014; Desmond et al. 2015, Cines 2015).

Concerns for potentially more serious adverse events, including thrombosis, conversion to malignancy, increase in bone marrow reticulin/bone marrow fibrosis, and, particular to eltrombopag, cataracts and transaminitis, prompted a mandatory Risk Evaluation and Mitigation Strategy (REMS) surveillance program in the United States upon regulatory approval of romiplostim and eltrombopag. This surveillance was unrevealing for new or more frequent than predicted adverse events and was eased after approximately 3 years, in 2011 (US Food and Drug Administration 2016).

Emerging evidence suggests that ITP itself is a pro-thrombotic state with a twofold increased risk of venous thromboembolism and 50 % increased risk of arterial thrombosis (Langeberg et al. 2012). Since both romiplostim and eltrombopag have been shown to cause dose-dependent thrombocytosis, the risk of related thromboembolic events might increase. Clinical trials in adults with chronic ITP treated with romiplostim have demonstrated a 2–6.5 % incidence of thromboembolic events (TEE) (Kuter et al. 2008, 2010). In the pivotal clinical trial of romiplostim in adults with chronic ITP, 2/83 in the treatment group developed arterial embolism, while 1/42 patients in the placebo group developed a venous thromboembolism. Both patients with arterial thrombosis had a previous history of vascular disease (Kuter et al. 2008). In long-term follow-up, romiplostim did not demonstrate an increase incidence of thrombosis, and most events were similar to the most common events that occur in the general population (myocardial infarct, transient

Table 3 Adverse events of interest reported in key clinical trials of TRAs in adults with ITP

Adverse event	Romiplostim	Eltrombopag	Avatrombopag
Reticulin fibrosis	6.9 %	8 %	NR
Cataracts	NR	1–2 %	NR
Thrombosis	2–6.5 %	2–5 %	6 %
Hepatic transaminitis	NR	7–10 %	3 %

NR not reported

ischemic attacks, deep vein thrombosis, and pulmonary embolism). There were, however, four cases of portal vein thrombosis in the treatment group vs. no portal vein thrombosis in the placebo treatment group (Cines 2015). In adults with chronic ITP treated with eltrombopag, studies have demonstrated an incidence of 2–5 % for TTE (Cheng et al. 2011; Saleh et al. 2013). In long-term follow-up in which the incidence of TEE was 5 %, all patients who experienced a TEE had at least one preexisting risk factor (smoking, hypertension, obesity, family history, or genetic predisposition), and 3 % had a previous history of TEE. Both venous and arterial thromboses were seen. The occurrence of TEE did not appear to correlate to platelet count, and some of the patients had platelet counts less than the treatment goal when the event occurred (Saleh et al. 2013). Treatment of adults with chronic ITP with avatrombopag has demonstrated a 6 % incidence of TEE. Again, both venous and arterial TEEs were seen. Three of the four patients who had a TEE had at least one preexisting risk factor (Bussel et al. 2014a, b).

Bone marrow fibrosis, mostly characterized by marrow reticulin accumulation, associated with the treatment with TRAs has been a concern in several trials with severity ranging from MF-1 to MF-3 on the European Consensus Scale (Mufti et al. 2006; Ghanima et al. 2014). Reticulin is a normal matrix component in the bone marrow, and up to 81 % of the general population has been shown to have some level of reticulin bone marrow fibrosis as high as MF-1 (Bauermeister 1971; Beckman and Brown 1990). Increased bone marrow reticulin has also been demonstrated in up to 40 % of adults with ITP not treated with TRAs (Ettrup et al. 2010). In the EXTEND study, which assessed long-term follow-up of adults with chronic ITP treated with eltrombopag, 8 % demonstrated MF-2 reticulin levels (Saleh et al. 2013). None demonstrated MF-3. Of the 147 patients who underwent bone marrow biopsy, only 11 received repeat biopsy after >2 years of treatment. Of the 11 patients, 8 showed stable levels of reticulin, 2 had a regression from MF-2 to MF-0, and 1 increased from MF-2 to MF-3 (Saleh et al. 2013). In the most recent report to date, 5-year follow-up data from the EXTEND trial showed none to mild reticulin in 98 % of the study participants. All patients have been asymptomatic and none have demonstrated conversion to a malignant process (Brynes et al. 2015). In a 3-year prospective study of adults with chronic ITP receiving romiplostim, 6.9 % (9 of 131) developed either a ≥2-grade increase in bone marrow reticulin (7 of 131) or collagen fibrosis (2 of 131) (Janssens et al. 2014). Earlier studies of romiplostim in chronic ITP have reported a range of reticulin fibrosis occurrence but lack long-term surveillance. In a single-center, partially retrospective study, using the European Consensus Grading System, 15 of 66 pediatric (24 %) or adult (76 %) patients with chronic ITP developed some degree of marrow fibrosis while receiving one of the three TRAs mentioned above. Interestingly, the outcome of fibrosis did not consistently progress with continuation of the TRA treatment. Data on reticulin or collagen fibrosis associated with TRA use remain limited. Further prospective surveillance is warranted.

Rebound worsening thrombocytopenia has been seen after cessation of therapy in adult patients with chronic ITP, although the rate of rebound is similar to that of placebo. In most cases, the platelet count returned to normal with or without rescue treatment and without sequelae (Bussel et al. 2006; Saleh et al. 2013). Monitoring of platelet counts for 4 weeks after cessation of therapy is recommended.

Eltrombopag is associated with hepatobiliary laboratory abnormalities (HBLA). In a minority of adults with chronic ITP, HBLA occurs at a rate of 7–10 % (Bussel et al. 2007; Cheng et al. 2011; Saleh et al. 2013). The EXTEND trial in adults with chronic ITP demonstrated a 10 % incidence of HBLA that did not correlate with dose or duration of therapy and most recovered with or without cessation of treatment. Those who had HBLA in a previous study that lead to cessation of treatment were retreated with eltrombopag in this follow-up study and did not have reoccurrence of HBLA. The abnormalities that have been seen are mild and reversible, and patients have been asymptomatic. In addition, an association between eltrombopag and hepatic decompensation in patients undergoing treatment for thrombocytopenia secondary to hepatitis C has been observed. Patients who received eltrombopag in addition to antiviral therapy had a higher rate of ascites and encephalopathy as compared to those receiving placebo and antiviral therapy (7 and 4 % in phase III trials and 10 and 5 % in open-label follow-up) (Afdhal et al. 2014). Monthly monitoring of liver function is recommended for all patients undergoing treatment with eltrombopag, with discontinuation of therapy if serum aminotransferase concentrations increase to >3x the upper limit of nomal concentrations, >3x above the patient's baseline concentrations with a progressive increase, sustained elevated aminotransferase concentrations for at least 4 weeks. Discontinuation of eltrombopag is also recommended for those patients with symptoms of chemical hepatitis. (US Food and Drug Administration 2016).

An association between TRAs and immunoblast proliferation is a potential untoward effect of hematologic growth factors. This has been of particular concern when TRAs are used in the treatment of myelodysplastic syndrome (MDS) (Giagounidis et al. 2014). TRAs do not appear to lead to MDS or leukemia in ITP (Saleh et al. 2013).

There has been an observation of cataracts in adults with chronic ITP treated with eltrombopag with an incidence of 0–8 % in the treatment population over a 6–26-week treatment course. Of note, in the RAISE study (a phase III

clinical trial), while 8 % of the treatment population developed cataracts, 10 % of the placebo group was also affected and in long-term follow-up of 3 years. Also, most patients had a history of earlier or concomitant treatment with corticosteroids, which may have contributed to the onset of or progression of the cataracts (Bussel et al. 2009; Cheng et al. 2011; Saleh et al. 2013).

In children, adverse events have been similar to those seen in adults (Elalfy et al. 2011; Ramaswamy et al. 2014). In children with chronic ITP treated with romiplostim, the most common adverse effects were headaches (35–63 %), pyrexia (24–54 %), and oropharyngeal pain (24–50 %) and with eltrombopag, nasopharyngitis (17 %), rhinitis (16 %), and epistaxis (13 %). Thromboembolic events may occur at a lower rate in children than in adults as no cases have been reported in randomized clinical trials of romiplostim or eltrombopag in children (Bussel et al. 2014a, b, 2015; Grainger et al. 2015; Tarantino et al. 2015a, b). Although surveillance bone marrow examination has not been part of the protocol in pediatric trials of the TRAs, the highest level or reticulin seen to date is MF-1, based on a recent retrospective study by Ramaswamy et al. (Ramaswamy et al. 2014). HLAB has been similarly occurring in approximately 6 % of children with ITP treated with eltrombopag. New or progression of existing cataract has occurred in 2 %. Again, all patients were also treated with corticosteroid which is a potentially confounding factor (Grainger et al. 2015).

Second-Generation TRAs in the Treatment of Other Thrombocytopenic Disorders

Liver Cirrhosis and Hepatitis C Infection

In patients with hepatitis C virus (HCV) infection, thrombocytopenia is a complication of chronic liver disease, HCV infection itself, as well as a known side effect of antiviral therapy. Thrombocytopenia in this patient population can lead to reduced or missed dosing of antiviral treatment which can lead to a lower rate of sustained antiviral response. In addition, surgery is often needed, but contraindicated in this population, due to high risk of bleeding secondary to thrombocytopenia. In the treatment of thrombocytopenia secondary to HCV infection and liver cirrhosis, TRAs have been shown to increase platelet count throughout treatment allowing for fewer dose reductions in antiviral therapy, increased sustained viral response rates, and completion of surgery without increase in bleeding or thrombotic events (McHutchison et al. 2007). There has been concern for increase in hepatic decompensation when eltrombopag is used in combination with interferon and ribavirin particularly in those with an albumin <5 g/dL and/or MELD score >10. An increase in thrombotic events such as portal vein thrombosis has also been seen in this study population (Afdhal et al. 2014; Giannini 2015; Moussa and Mowafy 2013).

Severe Aplastic Anemia

Severe aplastic anemia (SAA) is a disorder of pancytopenia and hypocellularity of the bone marrow. It is most often an autoimmune process that affects hematopoietic stem cells (HSCs) and progenitor cells. The complications of severe aplastic anemia are secondary to the cytopenias, primarily infection with neutropenia, and bleeding secondary to thrombocytopenia. Anemia often leads to the need for packed red blood cell transfusion (Desmond et al. 2015). Granulocyte colony-stimulating factor (G-CSF) and erythropoietin (EPO) have not been effective at treating aplastic anemia as they primarily act on their respective committed progenitor cells. Since the defect in SAA is at the HSC level, the ineffectiveness of G-CSF and EPO for the treatment of AA is somewhat expected. On the other hand, EPO binds and stimulates both megakaryocytes and HSCs. Eltrombopag also acts on both HSCs and megakaryocytes. In aplastic anemia, not only has eltrombopag been shown to increase platelet count, but it has also been found to exert a tri-lineage effect with an overall response rate of 44 % in at least one lineage. It also has decreased the need for transfusion in two clinical trials (Olnes et al. 2012; Desmond 2014). Despite the increase in cell counts, no change in bleeding complications or infections has been seen (Desmond 2014). The ultimate role of eltrombopag and other TRAs in the management of SAA is yet to be determined.

Myelodysplastic Syndrome

Myelodysplastic syndrome (MDS) is a heterogenous group of blood disorders characterized by bone marrow dyscrasia with clonal hematopoiesis, dysplastic bone marrow cells, various combinations of peripheral cytopenias, and increased risk of progression to acute myeloid leukemia. The cause of thrombocytopenia in MDS is multifactorial. Megakaryocytes are affected in several ways including exaggerated apoptosis and production of dysplastic, small, hypolobulated cells leading to impaired platelet production. To a lesser extent, enhanced platelet destruction may contribute to thrombocytopenia (Houwerzijl et al. 2006). MDS-associated thrombocytopenia may also be treatment related that can lead to dose reduction or missed doses of therapy and subsequently poorer outcomes. Thrombocytopenia occurs in approximately 40–80 % of patients with MDS. Currently, prognosis is predicted by the International Prognostic Scoring System Revised (IPSS-R) which uses a point system with higher scores indicating poorer prognosis and a maximum score of 10. The IPSS-R gives 0.5 points for

a platelet count 50–100 $\times$ 10^9/L and 1 point for a platelet count $<50 \times 10^9$/L . Platelet count, the need for platelet transfusion, and possibly the rate of platelet decline to $<50 \times 10^9$/L are independent prognostic indicators (Santini and Fenaux 2015). Currently, risk-based therapy is used and the goal of lower-risk MDS is to decrease the need for transfusion (Garcia-Manero 2015).

Romiplostim has been trialed for the treatment of thrombocytopenia in mild-to-intermediate myelodysplastic syndrome and has demonstrated a reduction in significant bleeding events and need for platelet transfusion per-patient-months, as well as being associated with no increase in thrombotic events. Increase in progression to AML is an obvious concern as this has been a potential adverse effect of interest in patients on TRAs without MDS; however, increase in progression to AML has not been reported. Despite an increase in the platelet count, improvement in mortality rate has not yet been shown with this mode of treatment. Those that have converted to AML while on TRAs have had WHO-defined refractory anemia with excess blasts type 1 or 2. The authors concluded that romiplostim should only be considered for mild-intermediate MDS (Prica 2014).

Romiplostim has also been trialed as an adjunct to other treatments such as lenalidomide, decitabine, and azacitidine. Results have shown a decreased need for reduction in dose of other treatments (Kantarjian et al. 2010; Wang et al. 2012; Greenberg et al. 2013). In vitro studies of eltrombopag in combination with lenalidomide in high-risk cell lines demonstrate inhibition of clonal expansion and inhibition of lenalidomide antiplatelet production effects on megakaryocytes, and eltrombopag alone, and in combination therapy, did not cause progression to leukemia. Eltrombopag alone has been shown to increase megakaryopoiesis at all stages without risk of blast cell formation in vitro (Will et al. 2009; Mavroudi et al. 2011; Tamari 2014).

Cytotoxic Chemotherapy-Induced Thrombocytopenia

Chemotherapy-induced thrombocytopenia (CIT) is dependent upon the type of chemotherapy agent used and occurs via a variety of mechanisms including the inhibition of platelet production, increased platelet destruction, and enhanced platelet clearance. The risk of hemorrhage in patients with CIT is directly related to the degree of thrombocytopenia, which often creates the need for platelet transfusion (which has its own risks), and missed or reduced dosing of chemotherapy (Kuter 2015). First-generation TRA use lead to higher nadir with early peak, reduced platelet transfusions, decreased duration of thrombocytopenia, decreased in missed chemotherapy dose, and possibly even increased survival (Fanucchi et al. 1997; Vadhan-Raj et al. 2000; Moskowitz et al. 2007). Romiplostim and eltrombopag have been shown to stimulate megakaryopoiesis, improve platelet counts (and in some cases, maintain platelet count $>100 \times 10^9$/L), and decrease rate of missed or reduced chemotherapy (Jeong et al. 2015; Winer 2014; Parameswanan 2014). Similar to studies completed in aplastic anemia, eltrombopag has demonstrated decreased anemia and neutropenia as well (Winer 2014). Ongoing studies are investigating novel uses for TRAs including treatment of chemotherapy-induced thrombocytopenia in solid tumors (Kellum et al. 2010), non-Hodgkin's lymphoma, and non-small cell lung cancer, persistent thrombocytopenia following hematopoietic stem cell transplant, and umbilical cord blood transplant. A list of currently approved indications for romiplostim and eltrombopag, at the time of this writing this article, is presented in Table 4.

Table 4 Approved Indications for romiplostim and eltrombopag

Indication	Romiplostim	Eltrombopag/Revolade
Chronic ITP, adults	USA, EU, Australia	USA, EU, Canada, Japan, Canada, Mexico, Brazil, Colombia, Turkey, UAE, Oman, Lebanon, Kuwait, Saudi, Egypt, Morocco
Chronic ITP, children (1–18 years)	–	USA, EU
Thrombocytopenia in HCV infection	–	USA, EU
Severe aplastic anemia	–	USA, EU

Inherited Thrombocytopenias

Among the inherited thrombocytopenias, MYH-9 related disease (MYH-9RD) is an uncommon macrothrombocytopenia characterized, additionally, by the presence of Dohle-like inclusion bodies in leukocytes, normal megakaryocytes, and variable findings of sensorineural hearing loss, presenile cataracts, and a proteinuric nephropathy. The MYH-9 gene codes for nonmuscular myosin IIA, a protein that is responsible for various cytoskeletal elements involved in motility. Thrombocytopenia in this disease is thought to be due to abnormal platelet release (Pecci et al. 2010). Platelet counts are highly variable between individuals, but most have thrombocytopenia, and most often the platelet count remains lower than normal, but stable throughout their lifetime. The most common bleeding abnormalities include epistaxis, menorrhagia, and easy bruising. Serious adverse bleeding events are rare. In premenopausal women, the most common complication is iron deficiency anemia due to menorrhagia. Bleeding risk is directly related to platelet count and spontaneous

bleeding is rare with platelet count $>50 \times 10^9$/L. Treatment is rarely needed; however, given the paucity of prospective studies and the rarity of the disease, the appropriate treatment of spontaneous bleeding and/or pre- and postoperative management is unknown. Eltrombopag has been successfully used in the preoperative period and was successful at raising the platelet count and allowing patients to participate in invasive surgery (Pecci et al. 2010). No bleeding complications were seen in the pre- or postoperative period. Also of interest, no increase in cataracts was seen.

Wiskott-Aldrich/X-Linked Thrombocytopenia

Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) are a spectrum of disorders caused by a mutation in the WAS gene. The WAS gene is exclusively expressed in hematopoietic stem cells, and its gene expression product is involved in cell signaling and cytoskeleton reorganization. WAS/XLT-related thrombocytopenia is characterized by small platelet volume, normal megakaryocyte number, and otherwise normal platelet morphology (Haddad et al. 1999; Kajiwara et al. 1999). Patients with WAS/XLT often experience spontaneous hemorrhage including gingival, gastrointestinal, and intracranial hemorrhage. Up to 30 % have a severe hemorrhagic event in their lifetime and 4–10 % of these episodes are fatal (Sullivan et al. 1994; Imai et al. 2004). While thrombocytopenia remains poorly understood in this disease, current hypotheses include increased platelet destruction (immune and nonimmune), decreased platelet size, and a decrease in platelet function (Marathe et al. 2009; Shcherbina et al. 2009; Gerrits 2015). Current palliative treatment options include platelet transfusion and splenectomy, with the only curative therapy being HSC transplantation. Eltrombopag has been shown to increase platelet count but no improvement in platelet function has been seen (consistent with pharmacokinetic studies as noted previously) with a subsequent decrease in bleeding (Gerritis 2015).

Summary

Since the discovery of megakaryocytes in the early 1900s, our understanding of platelet production and the pathophysiology of thrombocytopenia in various disease states has broadened. Despite the positive results seen with the recombinant TRAs, their induction of TRA and endogenous TPO antibodies rendered them useless. However, the development of the nonhomologous, apparently non-immunogenic TRAs, romiplostim and eltrombopag, has created potential treatment options for many thrombocytopenic disorders. Although initial registration studies focused on patients with chronic ITP, the second-generation TRAs, romiplostim and eltrombopag, have potential utility in inherited thrombocytopenias, CIT, MDS, and other secondary thrombocytopenias. As with other hematopoietic growth factors, surveillance for toxicity signals, including reticulin fibrosis and venous and arterial thrombosis, must continue.

Take Home Messages

1. Thrombopoietin (TPO) is the primary regulator of platelet production. Expression of the TPO is relatively constant.
2. Recombinant TPO and second-generation TPO receptor agonists, or TRAs increase the circulating platelet count in patients with a variety of thrombocytopenic conditions.
3. Two TRAs, romiplostim and eltrombopag, are approved in several countries for the treatment of chronic ITP (both), aplastic anemia (eltrombopag), thrombocytopenia related to liver disease (eltrombopag).
4. The safety profile of romiplostim and eltrombopag is largely good, however close monitoring for uncommon drug-specific adverse effects, such as bone marrow reticulin deposition or thromboembolic events, is recommended.

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Gene Therapy

Qizhen Shi and Robert R. Montgomery

Abstract

Gene therapy is an attractive approach for disease treatment. Since platelets are the second most abundant cells circulating in blood with the abilities of storage and delivery, it could be a unique target for gene therapy of diseases, e.g., hemophilia, in which platelets are involved. Recent studies have demonstrated that ectopic expression of factor VIII (FVIII) in platelets under control of the platelet-specific promoter results in FVIII storage together with its carrier protein von Willebrand factor (VWF) in α-granules and the phenotypic correction of hemophilia A. Importantly, the storage and sequestration of FVIII in platelets appears not only sufficient to selectively deliver FVIII to sites of hemostatic activation but in addition protects FVIII hemostatic bioactivity from function-blocking inhibitory antibodies. When factor IX (FIX) expression is targeted to platelets, FIX is also stored in α-granules and platelet-derived FIX restores hemostasis in hemophilia B mice, but the clinical efficacy is limited in the presence of anti-FIX inhibitory antibodies. In this chapter, recent studies utilizing platelets as a target for gene therapy of hemophilia A and B are reviewed.

Introduction

Genetically engineering of cells to produce proteins of interest is an appealing approach for disease treatment. The distinct facets of platelet biology (Elzey et al. 2003; Gawaz et al. 2005; Smyth et al. 2009; Semple et al. 2011; Jenne et al. 2013) make platelets a unique target for gene therapy (Yarovoi et al. 2003; Wilcox et al. 2003; Shi et al. 2006, 2007, 2008, 2014, 2015; Kuether et al. 2012; DU et al. 2013; Chen et al. 2014; Greene et al. 2014; Wang et al. 2015). Platelets circulating in blood through the whole body are packed with abundant secretory granules. These granules contain a large number of proteins from both megakaryocyte synthesis and endocytosis. When platelets are activated, the bioactive proteins are released from their granules, participating in a variety of physiologic and pathologic processes. Given the capacities of storage, trafficking, and release, utilizing platelets to deliver therapeutics is an attractive approach for disease treatment. In this chapter, recent studies using platelets as a means to deliver coagulation factors for hemophilia gene therapy are reviewed.

Platelet-Specific Genetic Manipulation

Gene therapy is a cutting-edge promising approach for the treatment of a number of diseases, such as genetic-inherited disorders and certain cancers. Gene therapy is the delivery of

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nucleic acid to correct a defective gene or introduce novel therapeutic genes into cells as a means to treat disease, which might lead to a cure. For gene therapy to be successful, a careful choice of the targeted cells for stable transgene expression and an efficient gene delivery vector are critical.

The Target Cell for Gene Transfer for Platelet-Specific Gene Therapy

For platelet-specific gene therapy of hemophilia, platelets are the final target for transgene protein expression and storage. Although platelets have components required for mRNA translation and protein synthesis (Booyse and Rafelson Jr. 1968; Booyse et al. 1968; Ts'ao 1971; Belloc et al. 1982; Kieffer et al. 1987) and de novo proteins are translated in response to cellular activation during physiologic or pathologic processes (Booyse and Rafelson Jr. 1967; Warshaw et al. 1967; Belloc et al. 1982; Kieffer et al. 1987; Weyrich et al. 2004), platelets do not have nuclear transcriptional capacity because they are anucleate cells. In addition, platelets have a limited life span of 8–10 days in humans (Harker et al. 2000; Brown et al. 2000; Scull et al. 2010) and 4–5 days in mice (Harker 1977; Nayak et al. 2013). Thus, direct molecular manipulation of platelets cannot serve as a reliable means for intervention. While megakaryocytes, which are the precursors of platelets, are amenable for genetic manipulation, they also have a limited life span and cannot serve as a means for long-term transgene expression.

In contrast, hematopoietic stem cells (HSCs) are preferable targets for gene transfer to establish in vivo long-term transgene expression in platelets because HSCs have the ability to self-renew and are capable of differentiating into all blood lineages including the megakaryocyte/platelet lineage (Johnson et al. 1982; Srour et al. 1991; Weissman 2000; Lemischka and Moore 2003). Thus, targeting transgene into HSCs may result in transgene protein expression in platelets throughout the lifetime of the recipients. In addition, there are many other aspects that make HSCs a favorable target for gene transfer. For example, HSCs are easily accessible. They can be expanded and genetically manipulated ex vivo followed by reimplantation. HSCs can be obtained not only from natural sources such as bone marrow and umbilical cord blood but also from peripheral blood after mobilization (Indovina et al. 1990; Ings et al. 2006; Ozkan et al. 2015; Karakukcu and Unal 2015). Recent studies showed that induced pluripotent stem (iPS) cells can be differentiated into hematopoietic progenitors (Schenke-Layland et al. 2008; Lengerke et al. 2009; Niwa et al. 2009; Tashiro et al. 2012), suggesting that iPS cells could be a potential source for HSC production. Although efficacy and safety are still issues, with the advanced techniques and increasing knowledge of stem cell biology, generating HSCs from the iPS cells may become possible in the future. Furthermore, from safety point of view, ex vivo transduction followed by transplantation of transduced HSCs to introduce transgene expression should minimize potential antiviral vector immune responses and germ line transmission of vector genomes in contrast to in vivo systematic transduction.

The Promoters Used to Restrict Transgene Expression to the Megakaryocyte/Platelet Lineage

Although both tissue-specific gene promoters and ubiquitous promoters can be used to drive transgene expression in platelets, there are advantages to utilizing tissue-specific promoters to restrict transgene expression to the platelet lineage. Using a tissue-specific promoter, which is a normal cellular gene promoter, rather than a strong viral promoter, to direct therapeutic transgene expression under physiologic regulation may reduce the risks of activation of adjunct cellular genes, thus reducing potential for insertional oncomutagenesis and genome toxicity. In addition, using tissue-specific promoters to restrict transgene expression to the platelet lineage may reduce potential immune response to transgene protein because the platelet-specific promoters will remain inactive in non-platelet lineages, thus reducing potential to activate the immune system. Evidence suggests that immune responses to transgene products are primed by antigen-presenting cells (APC) that are transduced and express transgene antigens (Corr et al. 1996; Doe et al. 1996; Ciesielska et al. 2013). Using tissue-specific promoters to direct transgene expression may avoid transgene expression in APC and thereby abrogate undesired immune responses against transgene product.

To limit transcription of the transgene to the platelet lineage, a megakaryocyte/platelet lineage-specific promoter is required to control the transgene expression. Theoretically, any promoter that directs the transcription of genes encoding a platelet-specific protein can be used to restrict transgene expression to platelets. With a better understanding of the characteristics of promoters, it is becoming more feasible to use a tissue-specific promoter to direct transgene expression to specific tissues. However, we should keep in mind that the efficacy and specificity of transgene expression under control of a manipulated promoter might not be exactly the same as endogenous gene expression because many elements can influence transgene expression. These include the region of the promoter used (DU et al. 2013), the transgene integration site (Clark et al. 1994) and copy number (Garrick et al. 1998; Calero-Nieto et al. 2010), and other gene expression regulators, such as enhancers, silencers, insulators, matrix attachment regions, locus control regions,

and microRNA interactions (Pennacchio and Rubin 2001; Moreno-Moya et al. 2014). Thus, it is very possible to achieve various expression levels of transgene protein among individuals and individual cells within a single recipient even using the same expression cassette with the same promoter.

The promoters that have been used to restrict transcription of transgene to the megakaryocyte/platelet lineage include glycoprotein (GP) Ibα (Yarovoi et al. 2003; Ohmori et al. 2006, 2008; Wang et al. 2015; Arachiche et al. 2014), GPIIb (α_{IIb}) (Berridge et al. 1985; Tronik-Le et al. 1995; Poujol et al. 1998; Tropel et al. 1997; Fang et al. 2005; Shi et al. 2003, 2004, 2006, 2007, 2014; Wilcox et al. 1999, 2000; Rodriguez et al. 2002; Chen et al. 2014; Arachiche et al. 2014; Schroeder et al. 2014; Kanaji et al. 2014), GPV (Sato et al. 2000), GPVI (Ohmori et al. 2006), GPIX (Bastian et al. 1999), platelet factor 4 (PF4) (Ravid et al. 1991; Kufrin et al. 2003; Damon et al. 2008; Siner et al. 2013), and c-mpl (Deveaux et al. 1996; Kaushansky and Drachman 2002). All of these promoters are active in megakaryocytes and expression of transgene continues in their platelet progeny. Most in vivo studies have used either the α_{IIb} promoter or the GPIbα promoter for platelet-/megakaryocyte-targeted transgene expression. The α_{IIb} promoter is a well-characterized promoter that is active throughout megakaryocytopoiesis including in megakaryocyte progenitors as well as multipotent HSCs (Poujol et al. 1998; Prandini et al. 1996; Tronik-Le et al. 1995; Tropel et al. 1997; Uzan et al. 1995; Murray et al. 1996). Evidence shows that the α_{IIb} promoter is also active in primitive HSCs (Debili et al. 2001; Mikkola et al. 2003; Robin et al. 2011) and mast cells (Berlanga et al. 2005). In contrast, the GPIbα promoter is active at relatively later stages of megakaryocytopoiesis (Debili et al. 1992). It has been reported that the GPIbα is also expressed on endothelial cells (Sprandio et al. 1988; Konkle et al. 1990; Wu et al. 1997), dendritic cells (Monteiro et al. 1999), and breast carcinoma cell lines (Oleksowicz et al. 1997). The lineage-specific expression of the endogenous genes regulated by these promoters is summarized in Table 1.

The Vectors Used for Transgene Delivery

Efficient transfection and stable integration of transgene into the genome of HSCs are a key requisite for sustained transgene expression in megakaryocytes/platelets derived from these cells. Since most of the long-term repopulating HSCs are in a quiescent state, only integrating vectors that have the ability to transduce nondividing cells can efficiently introduce transgene into these cells. Although conventional retroviral vectors can integrate into the genome, they are not the best vectors for transgene delivery to HSCs. Retroviral vectors require cell division for integration (Lewis and Emerman 1994; Medin and Karlsson 1997; Purton et al. 2001), and they have a predisposition to integrate in regions close to the transcription start site of cellular genes,

Table 1 The lineage-specific expression of the endogenous gene products

	Hematopoietic progenitors	Megakaryocyte precursors	Mature megakaryocytes	Platelets	Other cells
GPIIb (α_{IIb})	Yes (Murray et al. 1996; Debili et al. 2001; Mikkola et al. 2003; Robin et al. 2011)	Yes (Rabellino et al. 1981)	Yes (Uzan et al. 1995)	Yes (Uzan et al. 1995)	Mast cells (Berlanga et al. 2005)
GPIbα		Yes (Roth et al. 1996)	Yes (Roth et al. 1996)	Yes (Roth et al. 1996)	Endothelial cells (Sprandio et al. 1988; Konkle et al. 1990; Wu et al. 1997), dendritic cells (Monteiro et al. 1999), breast carcinoma cell lines (Oleksowicz et al. 1997)
GPV		Yes (Roth et al. 1996)	Yes (Roth et al. 1996; Sato et al. 2000)	Yes (Roth et al. 1996; Sato et al. 2000)	
GPVI		Yes (Jandrot-Perrus et al. 2000)	Yes (Jandrot-Perrus et al. 2000)	Yes (Jandrot-Perrus et al. 2000)	Endothelial cells (Sun et al. 2003)
GPIX		Yes (Roth et al. 1996)	Yes (Roth et al. 1996; Bastian et al. 1999)	Yes (Roth et al. 1996; Bastian et al. 1999)	
PF4		Yes (Doi et al. 1987)	Yes (Doi et al. 1987)	Yes (Doi et al. 1987)	
c-mpl	Yes (Kaushansky and Drachman 2002)	Yes (Kaushansky and Drachman 2002)	Yes (Kaushansky and Drachman 2002)	Yes (Kaushansky and Drachman 2002)	Endothelial cells (Cardier and Dempsey 1998)

increasing risks of insertional mutagenesis (Wu et al. 2003; Mitchell et al. 2004; De et al. 2005; Cattoglio et al. 2010). Lentiviral vectors, which are derived from the human immunodeficiency virus (HIV), have advantages over retroviruses in that they target both dividing and nondividing cells including quiescent HSCs (Miller et al. 1990; Bukrinsky et al. 1993; Sutton et al. 1998; Zennou et al. 2000), and unlike retrovirus, they do not have genomic integration hot spots (Mitchell et al. 2004; Gonzalez-Murillo et al. 2008; Biffi et al. 2011; Ustek et al. 2012). With high transduction efficacy and low genotoxicity, lentiviral vectors represent a promising tool for gene therapy of many disorders via the use of HSCs as a target for genetic manipulation. Utilizing a lentivirus-mediated gene transfer system to introduce a transgene expression cassette under control of a platelet-specific promoter into HSCs could potentially provide patients with a self-replicating pool of stem cells for long-term transgene expression in megakaryocytes and platelets derived from the manipulated HSCs. This will establish an in vivo cellular site for therapeutic protein production. This, together with a preestablished storage capacity and delivery system, makes platelets a unique target for gene therapy.

Platelet-Specific Gene Therapy of Hemophilia A

Hemophilia A is a recessive X-linked bleeding disorder resulting from a factor VIII (FVIII) deficiency (Chavin 1984). Although protein replacement therapy is readily available and effective for patients with hemophilia A, it is expensive and the requirement of frequently accessing blood vessels often limits its universal availability (Wong and Recht 2011; Mannucci et al. 2012). Furthermore, up to 35 % of patients with severe hemophilia A will develop inhibitory antibodies that inactivate FVIII procoagulant activity after protein infusion (Iorio et al. 2010; Mancuso et al. 2012; Franchini et al. 2013). These antibodies, referred to as inhibitors, cause the clinical failure of routine replacement therapy for bleeding episodes (White et al. 1982; Scandella 2000; Furie et al. 1994). Immune tolerance induction by aggressive infusion of FVIII is a treatment option for the eradication of anti-FVIII inhibitors, but it is very expensive, is protracted, and may not always be effective (Astermark 2003; Wight et al. 2003; Knight et al. 2003; Bohn et al. 2004). Gene therapy is an attractive alternative for hemophilia A treatment because it may provide a cure if successful. Substantial progress has been achieved in the last two decades, but immune responses to viral vector or transgene protein are still major concerns (Evans and Morgan 1998; Kootstra et al. 2003; Ye et al. 2004; Gallo-Penn et al. 2001; Matsui et al. 2007, 2011; Zhang et al. 2011; High 2011). Gene therapy of hemophilia A with inhibitors could be more challenging because inhibitory antibodies in the circulation will inactivate functional FVIII if it is constitutively secreted into plasma.

The Impact of VWF on Hemophilia A Gene Therapy

FVIII is a large and relatively unstable plasma glycoprotein that performs an important role in the intrinsic pathway of blood coagulation (Mann et al. 1990). FVIII naturally circulates in an inactive form in plasma in a tightly bound, non-covalently linked complex with its carrier protein VWF (Wagner 1990). Formation of a VWF/FVIII complex is important for FVIII stability and survival. If FVIII does not bind to VWF, it is degraded rapidly by proteases in plasma (Weiss et al. 1977; Chuah et al. 1998; Wise et al. 1991; Kaufman and Pipe 1999). Therefore, it has been thought in the past that for gene therapy to be successful, FVIII must be secreted directly into circulation to allow rapid binding to circulating VWF. The lack of success of previous clinical gene therapy trials (Roth et al. 2001; Powell et al. 2003; Chuah et al. 2004) spurs us to reconsider the target site(s) for FVIII expression and its relationship to VWF biosynthesis.

VWF is a large multimeric glycoprotein that performs multiple functions in hemostasis. Besides chaperoning FVIII as a complex in circulating blood, VWF initiates platelet adhesion at sites of injury, promotes platelet aggregation, and binds to collagen to facilitate clot formation. In a milieu of anti-FVIII antibodies, VWF protects FVIII from inhibitor inactivation (Gawryl and Hoyer 1982; Jacquemin et al. 2000; Shima et al. 1993; Saenko et al. 1994; Jacquemin 2010). Inhibitory antibodies against FVIII are time dependent (Briet et al. 1984; Gadarowski Jr. et al. 1988) because of the association-disassociation interactions between VWF and FVIII and inhibitors. Thus, targeting FVIII expression to cells that synthesize VWF in vivo could be beneficial for gene therapy of hemophilia A and hemophilia A with inhibitors. It is well known that VWF is exclusively produced by megakaryocytes and endothelial cells and stored in the α-granules in megakaryocytes and platelets and Weibel-Palade bodies in endothelial cells (Nachman et al. 1977; Jaffe et al. 1973; Wagner 1993). The cellular site of FVIII synthesis, which had been controversial for decades, has finally become clear in 2014. It is concluded simultaneously by two groups utilizing tissue-specific knockout models that endothelial cells are the primary source of FVIII synthesis in mice (Fahs et al. 2014; Everett et al. 2014), although it is still uncertain which subsets of endothelial cells synthesize FVIII and whether FVIII is synthesized by the same subsets of cells synthesizing VWF. It is known with certainty that FVIII circulates as a complex with VWF in blood (Fig. 1). FVIII is not detected in human (Wilcox et al. 2003; Shi et al. 2014), canine (DU et al. 2013), or murine platelets (Yarovoi et al. 2003; Wilcox et al. 2003; Shi et al. 2014). To date, there is no evidence showing either FVIII synthesis or release from normal platelets.

Early studies of the in vitro production of recombinant FVIII demonstrated enhancement of FVIII synthesis when

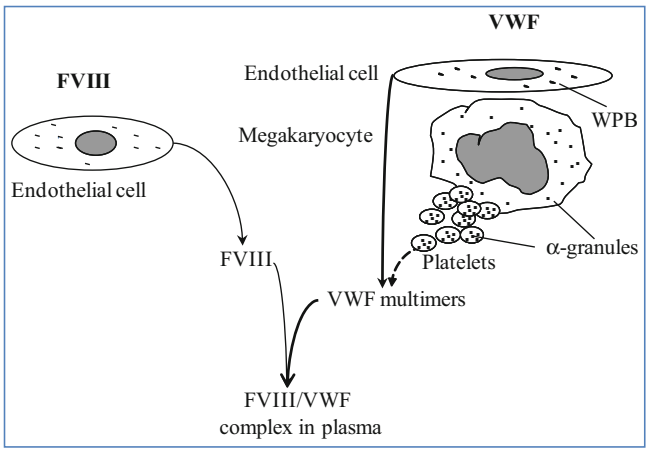

Fig. 1 The cellular sources of VWF and FVIII synthesis. VWF is synthesized by endothelial cells and megakaryocytes and stored in Weibel-Palade bodies in endothelial cells and α-granules in megakaryocyte and platelets. Recent studies demonstrate that FVIII is also synthesized by endothelial cells, but it is still unclear whether FVIII and VWF are produced in different subsets of endothelial cells. FVIII is bound to VWF in a complex circulating in plasma. Figure was used by permission of Q. Shi

performed in a cell that is also synthesizing VWF (Kaufman et al. 1988, 1989). Thus, directing FVIII expression to cells that synthesize VWF (megakaryocytes and endothelial cells) in vivo could potentially result in the formation of an intracellular VWF/FVIII complex and enhance the stability of FVIII compared to cells not synthesizing VWF (hepatocytes, fibroblasts). Targeting FVIII expression to platelets could be especially beneficial for gene therapy of hemophilia A because FVIII will be delivered together with VWF to the right place at the right time, where and when it is needed. This is particularly important for hemophilia A with inhibitors because FVIII would be sequestered by platelets, avoiding inhibitor inactivation in the circulation. Furthermore, a substantial amount of platelet-derived FVIII may be released at hemostatic sites where aggregated platelets become activated at sites of injury, thus circumventing the time-dependent inactivation by antibodies and achieving improved hemostasis (Fig. 2). Additionally, more tightly regulated release by platelets may limit immune exposure to the FVIII gene therapy product, reducing the incidence of inhibitor formation.

Proof of Principle Studies Using Transgenic Mouse Models

The hypothesis motivating platelet gene therapy of hemophilia A is that targeting synthesis of FVIII to megakaryocytes under control of a platelet-specific promoter will direct the storage of FVIII with VWF in the α-granules of platelets derived from these cells. This will establish a pool of FVIII together with VWF in platelets capable of stimulated release at the site of injury, avoiding inhibitor inactivation and achieving hemostasis. Several laboratories have been devoting efforts to develop unique gene therapy approaches using platelets as a target to deliver therapeutics for hemophilia A treatment, which are summarized below.

The αIIb Promoter-Driven Model

To restrict FVIII expression to the platelet lineage, Shi and co-workers have developed a vector, 2bF8, in which human B-domain deleted FVIII expression is driven by the platelet-specific α_{IIb} promoter. In ex vivo studies, Shi et al. found that FVIII expression in Dami cells, a megakaryocyte cell line, is greater when driven by the α_{IIb} promoter compared to the CMV promoter (Shi et al. 2003). When the 2bF8 expression cassette was used to generate transgenic mice on a FVIII knockout background ($2bF8^{tg}$) (Shi et al. 2006), FVIII was specifically expressed in platelets and stored together with endogenous murine VWF in platelet α-granules as determined by confocal microscopy and electron microscopy, and that platelet-derived FVIII is therapeutically effective even in the presence of high titers of anti-FVIII inhibitors. With no detectable plasma FVIII, the level of FVIII in platelets of heterozygous $2bF8^{tg}$ mice corresponded to about 1.4 % of FVIII in normal mouse whole blood. Remarkably, the therapeutic benefit of this platelet FVIII exceeded the benefit of 100 % plasma FVIII in the presence of inhibitors, using a tail-clip model (Shi et al. 2006).

Further studies by Shi et al. using $2bF8^{tg}$ mice have demonstrated that pre-existing immunity does not preclude 2bF8 genetically modified therapeutic engraftment when a sufficient preconditioning regimen is employed (Shi et al. 2008). The studies showed that even with only 1–5 % platelets containing FVIII, the bleeding phenotype was still significantly improved in hemophilia A mice with pre-existing anti-FVIII immunity. To explore the role of VWF in platelet gene therapy of hemophilia A, Shi et al. developed another transgenic mouse model by crossing 2bF8 transgene onto a VWF and FVIII double knockout background ($2bF8^{tg}/VWF^{-/-}$). The level of platelet-FVIII expression was decreased significantly, but the bleeding diathesis is still rescued in $2bF8^{tg}/VWF^{-/-}$ mice in the absence of antibodies in a tail-clipping injury model. However, the clinical efficacy was abrogated in the presence of inhibitors (Shi et al. 2015), demonstrating that VWF is critical in platelet gene therapy of hemophilia A with inhibitors.

The GPIbα Promoter-Driven Model

Yarovoi and co-workers (Yarovoi et al. 2003) have developed a transgenic model in which human B-domain deleted FVIII (hBF8) expression is directed by the platelet-specific

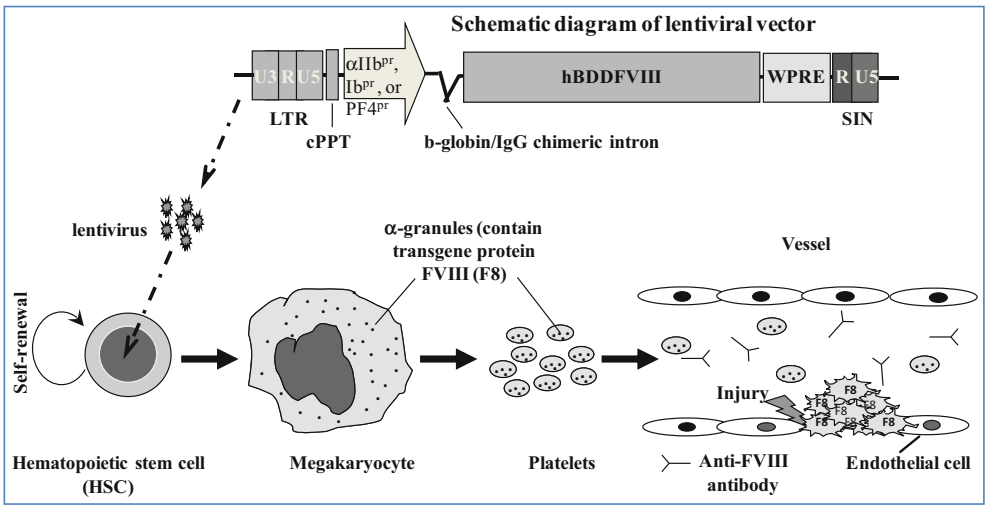

Fig. 2 Schematic diagram of platelet-specific gene therapy of hemophilia A. Lentiviral vectors harboring FVIII expression cassette under control of a platelet-specific promoter (α_{IIb}, Ib, or PF4 promoter) are used to transduce hematopoietic stem cells (HSCs). Transduced HSCs undergo self-renewal as well as differentiation into megakaryocytes where FVIII transgene protein will be made and stored in α-granules which will be shed into platelets circulating in blood. Platelet-sequestered FVIII will be protected from anti-FVIII inhibitory antibody inactivation. At the site of injury, FVIII (together with its carrier protein VWF) will be released from activated platelets, and thus time-dependent inhibitor activation may be circumvented, achieving improved hemostasis. Figure was used by permission of Q. Shi

GPIbα promoter (IbF8). In their highest-expressing hBF8 transgenic line, h38/F8^{null}, FVIII was detected in platelet releasates with a level corresponding to about 9 % of the antigen and 3 % of the activity in normal mouse plasma as determined by ELISA and Coatest assays. This platelet FVIII completely restored hemostasis in hemophilia A mice in a $FeCl_3$ carotid artery injury model, achieving an occlusive time similar to that obtained from wild-type animals. The cuticular injury model and whole blood clotting time confirmed that the hemophilic phenotype was improved in transgenic mice. Further studies by Yarovoi et al. demonstrated that FVIII is still stored in platelet α-granules even in the absence of VWF and that platelet-derived FVIII without VWF can still rescue the hemophilic phenotype in hemophilia A mice in the absence of inhibitors, and the clinical efficacy was similar to that in the presence of VWF although the level of platelet FVIII was significantly reduced (Yarovoi et al. 2005).

To compare the clinical efficacy of platelet FVIII to plasma FVIII, Gewirtz et al. infused a mixture of monoclonal antibodies into either IbF8 transgenic mice, line h38/F8^{null}, with platelet-FVIII expression or FVIII^{null} mice pre-infused with recombinant human FVIII (rhF8) (Gewirtz et al. 2008). Under those conditions, Gewirtz and co-workers found that platelet FVIII provided marginally better clot-forming activity in the $FeCl_3$-induced thrombosis model (Gewirtz et al. 2008). There is a difference in outcome between studies conducted by Shi et al. utilizing a tail-clipping injury model comparing the efficacy of platelet FVIII from 2bF8^{tg} mice and plasma FVIII in the presence of inhibitors (Shi et al. 2006, 2008) and those of Gewirtz and co-workers (Gewirtz et al. 2008). Studies by Shi et al. demonstrated that platelet FVIII was dramatically more effective than equivalent levels of infused FVIII when inhibitors were infused into the circulation prior to FVIII to mimic the clinical situation of FVIII protein replacement therapy in an inhibitor patient. Further studies showed that the different outcomes were driven by the order of infusions of rhF8 and inhibitors (Shi et al. 2012). Infusing of rhF8 into FVIII^{null} mice first followed by antibody infusion allows pre-association of infused FVIII with endogenous VWF in plasma into a protective VWF/FVIII complex before exposure to inhibitors. Even though pre-infusing rhF8 into FVIII^{null} mice followed by antibody infusion is not really quite analogous to the treatment of an inhibitor patient where FVIII is infused into a milieu of pre-existing polyclonal inhibitory antibodies, data from Gewirtz and co-workers' studies (Gewirtz et al. 2008) indicate that the preformed VWF/FVIII complex in platelets (intracellular) is more effective than the complex in plasma (extracellular) when faced with antibodies, suggesting that the properties of both platelets and VWF are important in platelet gene therapy of hemophilia A with inhibitors.

Another study by Neyman et al. using the IbF8 transgenic model, line h38/F8^{null}, showed that the clots formed in IbF8 transgenic mice had a tendency toward more embolization than those generated in FVIII^{null} mice receiving full-length recombinant FVIII infusion in a laser-induced cremaster vessel injury model (Neyman et al. 2008). Subsequently, Greene et al. developed additional transgenic lines in

which FVIII variants including IR8 (inactivation resistant F8) and cBF8 (canine B-domain deleted FVIII) were expressed under control of the same GPIbα promoter. The level of platelet FVIII in cBF8 transgenic mice was low, only 30 % of the hBF8 or IR8, but it significantly decreased clot embolization in the cremaster injury model (Greene et al. 2010).

The PF4 Promoter-Driven Model

Damon et al. generated a transgenic mouse model in which a rat PF4 promoter was used to direct human B-domain deleted FVIII expression (Damon et al. 2008). FVIII expression was confined to platelets and functional FVIII activity was detected by a chromogenic assay in the sonicated platelet lysates from transgenic mice with a level of 122 mU/10^9 platelets. Their studies demonstrated that platelet-derived FVIII is in an inactive form that requires thrombin cleavage for activation. Ectopic expression and storage of FVIII does not affect phosphatidylserine exposure nor α-granule release of endogenous platelet protein, e.g., factor V/Va. Their studies showed that infusion of thrombopoietin into transgenic mice resulted in a reduction of the amount of FVIII per platelet and clinical efficacy in hemostasis was completely abolished, although the total amount of circulating platelet FVIII was not affected. It is unclear why thrombopoietin-induced thrombocytopoiesis diminished the clinical efficacy of platelet-derived FVIII in this transgenic model.

Taken together, the studies using the transgenic approach provide proof of principle that platelet-specific expression could be successful for treating hemophilia A even in the presence of inhibitors.

Lentivirus-Mediated Platelet-Specific Gene Therapy of Hemophilia A

To apply the platelet-derived FVIII approach as a clinically translatable gene therapy model, efficient gene transfer and stable expression of FVIII are critical. Since HSCs are the preferable target for gene transfer and lentivirus can efficiently transduce stem cells, utilizing lentiviral vectors to introduce a FVIII expression cassette driven by a platelet-specific promoter should make it possible to establish long-term FVIII expression specifically in megakaryocytes/platelets.

Non-inhibitor Model Studies

Shi et al. used a lentivirus-mediated gene transfer system to deliver the 2bF8 expression cassette into HSCs, resulting in FVIII expression in platelets (Shi et al. 2007). These studies demonstrated that transplantation of 2bF8 lentivirus-transduced bone marrow cells into $FVIII^{null}$ mice preconditioned with 11Gy total body irradiation (TBI) resulted in sustained therapeutic levels of platelet FVIII and restored hemostasis with neither inhibitory nor non-inhibitory antibody development. The expression levels of platelet FVIII in transduced recipients (0.60 ± 0.26 mU/10^8 platelets) were similar to those obtained in $2bF8^{tg}$ mice, which were generated by embryonic stem (ES) cell-mediated transgenesis (Shi et al. 2006). Serial transplants demonstrated that platelet-FVIII levels were maintained after sequential bone marrow transplantation, confirming that long-term repopulating HSCs were successfully transduced. These studies build the foundation for lentivirus-mediated platelet-specific gene therapy.

Greene et al. developed lentiviral vectors harboring hBF8, IR8, or cBF8 under control of the GPIbα promoter and introduced FVIII expression by bone marrow transduction and transplantation into $FVIII^{null}$ mice preconditioned with 10Gy TBI (Greene et al. 2010). They found that the level of platelet-FVIII expression in transduced recipients was about 50 % of the transgenic mice with hBF8 or IR8 cassettes, but similar low levels were observed in both transgenic and transduced mice with the cBF8 cassette. Hemostasis was improved in transduced recipients, with better outcomes using IR8 and cBF8 than with hBF8. Further studies from the same group showed that the low level of FVIII expression in cBF8-transduced recipients was associated with greater megakaryocyte apoptosis than in hBF8-transduced animals. When the furin cleavage site in hBF8 was modified by substituting His for Arg of 1645, the hemostatic efficacy of platelet-derived hBF8 was significantly improved in the cremaster laser injury model (Greene et al. 2014).

Inhibitor Model Studies

To investigate whether lentiviral gene delivery to HSCs can efficiently introduce therapeutic levels of platelet FVIII in hemophilia A mice with pre-existing anti-FVIII immunity, Kuether et al. transduced enriched HSCs (Sca 1^+) cells from rhF8-immunized $FVIII^{null}$ mice with 2bF8 lentivirus followed by transplantation into rhF8-immunized littermates (Kuether et al. 2012). Sustained therapeutic levels of platelet-FVIII expression were obtained, while inhibitor titers declined with time. Hemostatic improvement in the treated animals was confirmed by the tail-clip survival test and the electrolyte-induced injury model. The level of platelet-FVIII expression in the inhibitor model was not significantly different from the non-inhibitor model (1.56 ± 0.56 mU/10^8 platelets vs. 1.46 ± 0.43 mU/10^8 platelets) when a myeloablative conditioning regimen of 11Gy total body irradiation (TBI) was employed. There was a wide range of FVIII expression levels among recipients, ranging from 0.36 mU/10^8 platelets to 6.18 mU/10^8 platelets. When LAM-PCR (linear amplification-

mediated PCR) was used to survey the proviral DNA integration sites in 2bF8-transduced animals, 39 genomic insertion sites were identified in six primary recipients. No overrepresented insertion sites were noted, although one (*Mds1*) corresponds to a common integration site for γ-retroviral vectors (Calmels et al. 2005) and another, *Arid1a*, is a proto-oncogene presented in the Mouse Retrovirus-Tagged Cancer Gene Database.

In this study (Kuether et al. 2012), Kuether et al. also evaluated the efficacy of non-myeloablative conditioning regimens, including sublethal TBI and busulfan chemotherapy, in platelet gene therapy. The levels of platelet FVIII in the group conditioned with a non-myeloablative TBI regimen (6.6Gy) were not significantly different from the group conditioned with myeloablative TBI (11Gy). Busulfan, an alkylating agent with potent effects on primitive hematopoietic cells, used as a conditioning alone resulted in therapeutic levels of platelet-FVIII expression in a 2bF8-transduced non-inhibitor model. However, in the inhibitor model, some form of additional immune suppression, e.g., antithymocyte globulin or a low dose of TBI, was required for efficacy. These studies demonstrated that 2bF8 lentiviral gene delivery to HSCs can introduce sufficient therapeutic levels of platelet-FVIII expression in hemophilia A mice even with pre-existing immunity, and importantly with non-myeloablative conditioning, suggesting that platelet gene therapy may be a promising strategy for gene therapy of hemophilia A even in the high-risk setting of pre-existing inhibitors.

Dog Model Studies

To further evaluate the efficacy of platelet gene therapy of hemophilia A, Du et al. applied a 2bF8 gene therapy protocol to hemophilia A dogs (DU et al. 2013). Autologous HSCs ($CD34^+$) were enriched from G-CSF (granulocyte colony stimulation factor) and SCF (stem cell factor)-mobilized peripheral blood of hemophilia A dogs, transduced with 2bF8 lentivirus (renamed as -889ITGA2B-BDDFVIII-WPTS in this study) ex vivo, and transplanted back into animals preconditioned with busulfan. Cyclosporine and mycophenolate mofetil were administered for about 90 days after transplantation to prevent immune responses. FVIII was detected in 2bF8 lentivirus-transduced dog platelets and was stored in α-granules even though no VWF is present in canine platelets (Nichols et al. 1995; Sanders Jr. et al. 1995). In this study, Du and co-worker also examined whether adding a truncated VWF propeptide, which is known to have a capacity to reroute unrelated secreted protein to a storage pathway (Haberichter et al. 2002), into the construct (-673ITGA2B-VWFSPD2-BDDFVIII-WPTS) would affect the expression and storage of FVIII in canine platelets. It appeared that FVIII was stored better in α-granules of platelets in animal that received -673ITGA2B-VWFSPD2-BDDFVIII-WPTS lentivirus-transduced HSCs as determined by electron microscopy, but the platelet-FVIII expression level was similar to that obtained in animals receiving regular 2bF8 lentivirus-transduced HSCs. The levels of platelet-FVIII expression ranged from 5 to 9 $mU/10^8$ platelets. The occurrence of severe bleeding episodes was prevented in all three dogs for at least 2.5 years after transplantation of 2bF8-transduced autologous HSCs. Similar to results in the unimmunized hemophilia A mouse model (Shi et al. 2007), no anti-FVIII antibodies were detected in transduced dogs. These data demonstrate that 2bF8 lentivirus-mediated HSC transduction followed by autologous transplantation can introduce sustained therapeutic levels of platelet FVIII resulting in hemostatic improvement in hemophilia A dogs. Whether the vector containing VWF propeptide would be beneficial for gene therapy of hemophilia A in other species including humans, in which platelets already contain endogenous VWF, will need to be further investigated.

Human Cell Studies

To evaluate the feasibility of 2bF8 lentivirus-mediated human platelet gene therapy for hemophilia A, Shi et al. developed an immunocompromised hemophilia A recipient mouse model [NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ FVIII^{null} (NSGF8KO)] and human cord blood (hCB)-derived HSCs were used as a target for gene transfer (Shi et al. 2014). $CD34^+$ hCB-derived HSCs were transduced with 2bF8 lentivirus ex vivo and *xeno*transplanted into NSGF8KO mice preconditioned with busulfan. Platelet FVIII was detected in all recipients for as long as human platelet chimerism persisted. Electron microscopy demonstrated that FVIII was colocalized with VWF in transduced human platelets. Tail-clipping tests and whole blood clotting time confirmed that hemostasis was improved in NSGF8KO mice that received 2bF8-transduced human HSCs (hHSCs). When nonrestrictive LAM-PCR was used to analyze 2bF8LV insertion sites, no identified integration sites were located within known proto-oncogenes in human hematopoiesis. Altogether, data from the human cell studies demonstrate the feasibility of 2bF8 lentiviral gene delivery to hHSCs to introduce FVIII expression in human platelets and that human platelet FVIII can improve hemostasis in hemophilia A mice.

In Vivo Selection to Enhance Platelet-FVIII Expression

With a goal of improving platelet-FVIII expression and reducing potential risks associated with platelet gene therapy, e.g., integrational mutagenesis and toxicities associated with preconditioning, Schroeder et al. developed a vector, 2bF8/MGMT, which harbors dual genes, the 2bF8 gene and a drug resistance gene, the MGMPTP140K cassette

(Schroeder et al. 2014). With this vector, platelet-FVIII expression can be induced by transduction of HSCs at a low MOI (multiplicity of infection) followed by transplantation into $FVIII^{null}$ mice preconditioned with a non-myeloablative regimen. The transduced cells can be enriched in vivo by treatment with O^6-benzylguanine (BG) followed by 1,3-bis-2 chloroethyl-1-nitrosourea (BCNU) which selectively kills untransduced hematopoietic cells, and thereby platelet-FVIII expression is enhanced. Even using a low MOI of 1 and sublethal 6.6Gy TBI, the level of platelet-FVIII expression in recipients after in vivo selection reached 4.3 ± 5.5 mU/10^8 platelets, which is 2.9-fold higher than that obtained from nonselectable 2bF8LV using an MOI of 10 with lethal 11Gy TBI (Kuether et al. 2012). When an MOI of 10 was used for transduction, the level of platelet-FVIII expression in BG/BCNU-treated transduced 2bF8/MGMT recipients reached 14.2 ± 12.1 mU/10^8 platelets. The highest platelet-FVIII expression was 35 mU/10^8 platelets, which corresponds to 70 % of FVIII:C in whole blood in normal mice. Phenotypic correction of the $FVIII^{null}$ coagulation defect in treated animals was confirmed by a tail bleeding test and ROTEM analysis of the whole blood clotting time. Importantly, there were no anti-FVIII antibodies detected in the BG/BCNU-treated transduced recipients even after rhF8 challenge, indicating that anti-FVIII immune tolerance is induced in treated animals.

Inducing Platelet-FVIII Expression Via In Situ Transduction of HSCs

To avoid preconditioning, Wang and co-workers (2015) developed a platelet gene therapy protocol through intraosseous delivery of a lentiviral vector, which harbors hFVIII/N6 [human FVIII with a partial B-domain, an N-terminal 226 amino acid stretch (N6)] cassette under control of a human GPIbα promoter (G-F8-LV) or a ubiquitous human elongation factor 1α (EF-1α) promoter (E-F8-LV), to transduce bone marrow cells in situ. Lentiviral vectors were directly injected into $FVIII^{null}$ mouse tibia through the join. The studies showed that 3–20 % FVIII activity was detected in plasma initially by a modified activated partial thromboplastin time (aPTT) assay in the animals receiving intraosseous infusion of E-F8-LV, but drop to undetectable levels within 2–3 months due to anti-FVIII immune response. In contrast, platelets containing FVIII were detectable in animals that received intraosseous infusion of G-F8-LV up to 160 days. FVIII antigen level was about 1 mU/10^8 platelets in the non-inhibitor model and 0.74 mU/10^8 platelets in the inhibitor model when 2.2×10^7 ifu/animal of G-F8-LV was infused. These studies demonstrate that platelet-FVIII expression can be achieved through in situ transduction of bone marrow cells when FVIII expression is restricted in platelet lineage.

Altogether, these studies demonstrate that induction of therapeutic levels of platelet-FVIII expression can be successfully achieved using lentivirus-mediated platelet-specific gene delivery to HSCs in hemophilia A even with pre-existing anti-FVIII immunity.

Platelet-Specific Gene Therapy of Hemophilia B

Hemophilia B is also an X chromosome-linked recessive bleeding disorder, which results from a factor IX (FIX) deficiency. It occurs at a frequency of 1/10,000 worldwide. Protein replacement therapy is effective, but it has similar problems as hemophilia A. Although the incidence of anti-FIX inhibitory antibody development is lower in hemophilia B patients after protein therapy, allergic reactions or anaphylaxis is very common in patients with inhibitors, limiting the use of protein infusion and increasing the risk of morbidity and mortality (Jadhav and Warrier 2000; Lusher 2000; Shibata et al. 2003; Franchini et al. 2009). Hemophilia B is a prime disease model for gene therapy. It is well known that FIX is a vitamin K-dependent protein that is normally synthesized by hepatocytes. Current clinical trials using adeno-associated virus serotype 8 (AAV8)-mediated liver-restricted FIX expression are very encouraging (Nathwani et al. 2011; Nathwani et al. 2014). Phase 1 trials showed that dose-dependent expression levels of FIX in circulation were achieved in severe hemophilia B patients after single dose vector administration and the bleeding diathesis was significantly improved. However, for individuals with severe liver disease or neutralizing antibodies to AAV, which are present in 30–50 % of the population (Calcedo et al. 2009; Calcedo et al. 2011), an alternative gene therapy approach might be desirable.

Studies from platelet-targeted FVIII expression have demonstrated that transgene FVIII is stored in platelet α-granules even in the absence of VWF. The hypothesis is that ectopic expression of FIX to platelets will also result in FIX storage in platelet α-granules and that stored FIX will be released locally at site of vascular injury to improve hemostasis in hemophilia B. To investigate the potential of platelet gene therapy of hemophilia B, Zhang and co-workers constructed a lentiviral vector (2bF9) in which hFIX expression cassette was under control of the α_{IIb} promoter and a transgenic model with platelet-specific FIX expression was generated by 2bF9 lentivirus-mediated transgenesis. Studies using this transgenic model demonstrated that 90 % of FIX is stored in platelet α-granules and is releasable by agonists and that platelet-derived FIX can rescue the bleeding phenotype in hemophilia B mice. Studies demonstrated that platelet FIX is completely carboxylated and has functional activity, indicating that megakaryocytes/platelets have the capacity to carboxylate the newly synthesized FIX protein

precursor to functional FIX protein. Hemostatic efficacy can be transferred by platelet transfusion or bone marrow transplantation. However, unlike platelet-derived FVIII, the hemostasis efficacy of platelet FIX is limited in the presence of anti-FIX inhibitors. The reason that platelet FIX behaves differently than platelet FVIII in the face of inhibitors could be the lack of a carrier protein to protect FIX as VWF protects FVIII, so antibodies can freely bind to FIX once they encounter each other in the plasma when FIX is released from activated platelets. This would be similar to 2bF8 on VWF and FVIII double knockout background. Indeed, without VWF, the clinical efficacy of platelet FVIII is abolished in the presence of anti-FVIII inhibitors (Shi et al. 2015).

Although platelet-derived FIX does not maintain clinical efficacy in the face of inhibitors, targeting FIX expression to platelets could still be a new strategy for gene therapy of hemophilia B. Chen and co-workers used 2bF9 lentiviral gene delivery to HSCs by transduction followed by transplantation to introduce platelet-specific FIX expression in hemophilia B mice (Chen et al. 2014). Sustained therapeutic levels of platelet-FIX expression were obtained in all recipients that received 2bF9-transduced HSCs. Flow cytometry analysis demonstrated that 6–39 % of platelets expressed FIX in transduced recipients, which was sufficient to rescue the bleeding diathesis in FIX^{null} mice in tail-clipping models, including the tail-clip survival test and a tail bleeding test. Sequential bone marrow transplantation demonstrated that platelet-FIX expression in secondary recipients was sustained, confirming that long-term engrafting HSCs were successfully modified. Importantly, none of the 2bF9-transduced recipients developed anti-FIX antibodies. Only one of nine recipients developed a low titer of inhibitors even after challenge with rhF9 in the presence of adjuvant. These data demonstrate that targeting FIX expression to platelets can not only restore hemostasis but also induce immune tolerance in hemophilia B mice, suggesting that platelet gene therapy may be a promising strategy for gene therapy of hemophilia B in humans.

The Safety Issues in Platelet Gene Therapy of Hemophilia

There are some concerns related to lentiviral gene delivery in platelet gene therapy. One major concern is the immune response to transgene product or viral proteins that may result in gene therapy failure because the transfected cells may be eliminated if cellular responses occur or the functional bioactivity of transgene protein may be inactivated if a humoral response is induced. It has been reported that immune responses are a problem in lentivirus-mediated HSC gene therapy when FVIII transgene expression was directed by ubiquitous promoters (Kootstra et al. 2003; Wang et al. 2015). This problem is not encountered when transgene expression is targeted to and stored in platelets. Instead, lentivirus-mediated platelet-specific gene therapy improves hemostasis but also induces immune tolerance in both hemophilia A and B mouse models (Schroeder et al. 2014; Chen et al. 2014).

The other significant concern is insertion site-related mutagenesis that is a potential risk associated with gene therapy utilizing any integrating vector. The advanced self-inactivating design of lentiviral vectors and the differences in integration site selection between lentiviral and oncoretroviral vectors may reduce the risk of insertional mutagenesis. Oncoretroviral vectors tend to integrate preferential nearby promoter-proximal regions, with hot spots in proto-oncogenes or genes related to cell proliferation. In contrast, lentiviral vectors appear to integrate more randomly into open chromatin without hot spots (Beard et al. 2007; Montini et al. 2006). While the studies utilizing LAM-PCR to survey 2bF8 integrants showed that there are no overrepresented insertion sites in animals that received 2bF8-transduced HSCs from mice (Kuether et al. 2012), dogs (DU et al. 2013), or humans (Shi et al. 2014), onco-mutagenesis resulting from random integration of transgene into the genome remains a potential risk in lentivirus-mediated gene transfer. Further studies using high-throughput techniques to more thoroughly determine the integration repertoire of these vectors are required.

In addition, the occurrence of abnormal on apoptosis in megakaryocytes when FVIII is ectopically expressed has been reported (Greene et al. 2014). Studies done by Poncz and co-workers showed that platelet-targeted FVIII expression, especially canine FVIII, under control of the GPIbα gene promoter increases apoptosis in transduced megakaryocytes, resulting in a 30 % reduction of platelet counts in transduced recipients (Greene et al. 2014). This does not seem to be the case when FVIII expression is driven by the α_{IIb} promoter. Studies by Schroeder et al. using the 2bF8/MGMT system have demonstrated that platelet number in lentivirus-transduced animals had fully recovered within 8 weeks after transplantation when a non-myeloablative conditioning regimen was applied and platelet counts were normal in transduced animals even with FVIII expression as high as 14.18 ± 12.05 mU per 10^8 platelets after in vivo drug selection, which corresponds to about 23 % of FVIII activity in normal mouse whole blood (Schroeder et al. 2014). It is unclear whether the cellular toxicity of FVIII expression in megakaryocytes is a specific phenotype driven by the GPIbα promoter, and therefore, further studies are warranted.

Whether ectopic expression of FVIII or FIX in platelets would have a potential thrombotic risk is also an important

safety issue that needs to be addressed before applying platelet gene therapy protocols to the clinic.

Summary

In summary, studies from preclinical trials using animal models have demonstrated the feasibility of platelet delivery of therapeutics for disease treatment through genetic manipulation of HSCs. Targeting FVIII or FIX expression and storage to platelets is a promising strategy for gene therapy, with potential to not only improve hemostasis but also induce immune tolerance in hemophilia. In a clinical scenario, patient-derived stem cells would be harvested from mobilized peripheral blood or cord blood for ex vivo introduction of a corrected FVIII or FIX gene driven by a platelet-specific promoter, followed by autologous transplantation. This is a promising approach for gene therapy of hemophilia A patients with inhibitors as well as non-hemophilic patients with acquired inhibitory antibodies who can also have life-threatening clinical bleeding which is difficult to treat by conventional therapies.

Take Home Messages

- Targeting FVIII or FIX expression and storage to platelets is a promising strategy for gene therapy, with potential to not only improve hemostasis but also induce immune tolerance in hemophilia.
- FVIII can be specifically expressed and stored together with its carrier protein VWF in platelets α-granules when it is driven by a platelet-specific promoter.
- Platelet-targeted FVIII expression can rescue the bleeding phenotype in hemophilia A even in the presence of anti-FVIII inhibitory antibodies.
- Without VWF, platelet-FVIII levels significantly decrease and the clinical efficacy is abolished in the presence of inhibitors.
- Platelet-targeted FIX expression can rescue the bleeding phenotype in hemophilia B mice, but the efficacy is limited in the presence of anti-FIX inhibitory antibodies.
- Platelet-specific gene therapy can induce immune tolerance in both hemophilia A and hemophilia B mice.

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